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The scientific basis and development of a matrix metalloproteinase (MMP) -8 specific chair-side test for monitoring of periodontal health and disease from gingival crevicular fluid

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Academic dissertation for the degree of PhD

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CONTENTS

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III. Kinane DF, Darby IB, Luoto H, Sorsa T, Tikanoja S, Mäntylä P. Changes in gingival crevicular fluid matrix metalloproteinase-8 levels during periodontal treatment and maintenance. J Periodontal Res 2003; 38: 400—404.

IV. Mäntylä P, Stenman M, Kinane DF, Tikanoja S, Luoto H, Salo T, Sorsa T. Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. J Periodontal Res 2003; 38: 436—439.

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VI. Mäntylä P, Stenman M, Paldanius M, Saikku P, Sorsa T, Meurman JH. *Chlamydia pneumoniae* together with collagenase-2 (MMP-8) in periodontal lesions. Oral Dis 2004; 10: 32—35.

2 ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
AL	attachment loss
ALP	alkaline phosphatase
α1-AT	alpha-1-antitrypsin
α2-MG	alpha-2-macroglobulin
AP	adult periodontitis
AST	aspartate aminotransferase
BANA	N-benzoyl-DL-arginine-2-naphthylamine
B. forsythus	*Bacteroides forsythus*
ß-G	beta-glucuronidase
ß-NAH	beta-N-acetyl-hexosaminidase
BI	bleeding index
BM	basement membrane
BOP	bleeding on probing
Ca	calcium
CAL	clinical attachment level
CAM	cell adhesion molecule
CA-MMP	cysteine array matrix metalloproteinase
CPITN	community periodontal index of treatment needs
C. pneumoniae	*Chlamydia pneumoniae*
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FcγR	Fc gamma receptor
GAG	glycosaminoglycan
GCF	gingival crevicular fluid
GI	gingival index
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
ICTP	pyridinoline cross-linked carboxyterminal telopeptide of type I collagen
IFMA	time-resolved immunofluorometric assay
Ig	immunoglobulin
IL	interleukin
IL-1ra	receptor antagonist of IL-1
JE	junctional epithelium
JP	juvenile periodontitis
kDa	kilo Dalton
LDD	low-dose doxycycline
LJP	localised juvenile periodontitis
LL-37	human cathelidicin peptide
LPS	lipopolysaccharide
MGI	modified gingival index
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MT-MMP	membrane-type MMP
NS	non-smoker
OC	osteocalcin
OPN	osteopontin

PCR	polymerase chain reaction
PGE_2	prostaglandin-E_2
PD	pocket probing depth
P. gingivalis	*Porphyromonas gingivalis*
PI	plaque index
PISF	peri-implant sulcus fluid
PMN	polymorphonuclear leukocyte, neutrophil leukocyte
PST	Periodontal Susceptibility Test
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
S	smoker
sICAM-1	intercellular molecule-1
SRP	scaling and root planing
Supp	suppuration
TNFα	tumour necrosis factor alpha
TIMP	tissue inhibitor of metalloproteinases
t-PA	tissue/blood vessel type plasminogen activator
u-PA	urokinase type plasminogen activator
VES	visual elastase scores
Zn	zinc

3 ABSTRACT

Matrix metalloproteinase (MMP) -8, collagenase-2, is a key mediator of irreversible tissue destruction in chronic periodontitis and detectable in gingival crevicular fluid (GCF). MMP-8 mostly originates from neutrophil leukocytes, the first line of defence cells which exist abundantly in GCF, especially in inflammation. MMP-8 is capable of degrading almost all extra-cellular matrix and basement membrane components and is especially efficient against type I collagen. Thus the expression of MMP-8 in GCF could be valuable in monitoring the activity of periodontitis and possibly offers a diagnostic means to predict progression of periodontitis.

In this study the value of MMP-8 detection from GCF in monitoring of periodontal health and disease was evaluated with special reference to its ability to differentiate periodontal health and different disease states of the periodontium and to recognise the progression of periodontitis, i.e. active sites. For chair-side detection of MMP-8 from the GCF or peri-implant sulcus fluid (PISF) samples, a dip-stick test based on immunochromatography involving two monoclonal antibodies was developed.

The immunoassay for the detection of MMP-8 from GCF was found to be more suitable for monitoring of periodontitis than detection of GCF elastase concentration or activity. Periodontally healthy subjects and individuals suffering of gingivitis or of periodontitis could be differentiated by means of GCF MMP-8 levels and dipstick testing when the positive threshold value of the MMP-8 chair-side test was set at 1000 μg/l. MMP-8 dipstick test results from periodontally healthy and from subjects with gingivitis were mainly negative while periodontitis patients' sites with deep pockets (> 5 mm) and which were bleeding on probing were most often test positive. Periodontitis patients' GCF MMP-8 levels decreased with hygiene phase periodontal treatment (scaling and root planing, SRP) and even reduced during the three month maintenance phase. A decrease in GCF MMP-8 levels could be monitored with the MMP-8 test. Agreement between the test stick and the quantitative assay was very good ($\kappa = 0.81$) and the test provided a baseline sensitivity of 0.83 and specificity of 0.96.

During the 12-month longitudinal maintenance phase, periodontitis patients' progressing sites (sites with an increase in attachment loss ≥ 2 mm during the maintenance phase) had elevated GCF MMP-8 levels compared with stable sites. General mean MMP-8 concentrations in smokers' (S) sites were lower than in non-smokers' (NS) sites but in progressing S and NS sites concentrations were at an equal level. Sites with exceptionally and repeatedly elevated MMP-8 concentrations during the maintenance phase were clustered in smoking patients with poor response to SRP (refractory patients). These sites especially were identified by the MMP-8 test.

Subgingival plaque samples from periodontitis patients' deep periodontal pockets were examined by polymerase chain reaction (PCR) to find out if periodontal lesions may serve as a niche for *Chlamydia pneumoniae*. Findings were compared with the clinical periodontal parameters and GCF MMP-8 levels to determine the correlation with periodontal status. Traces of *C. pneumoniae* were identified from one periodontitis patient's pooled subgingival plaque sample by means of PCR. After periodontal treatment (SRP) the sample was negative for *C. pneumoniae*. Clinical parameters or biomarkers (MMP-8) of the patient with the positive *C. pneumoniae* finding did not differ from other study patients.

In this study it was concluded that MMP-8 concentrations in GCF of sites from periodontally healthy individuals, subjects with gingivitis or with periodontitis are at different levels. The cut-off value of the developed MMP-8 test is at an optimal level to differentiate between these conditions and can possibly be utilised in identification of individuals at the risk of the transition of gingivitis to periodontitis. In periodontitis patients, repeatedly elevated GCF MMP-8 concentrations may indicate sites at risk of progression of periodontitis as well as patients with poor response to conventional periodontal treatment (SRP). This can be monitored by MMP-8 testing. Despite the lower mean GCF MMP-8 concentrations in smokers, a fraction of smokers' sites expressed very high MMP-8 concentrations together with enhanced periodontal activity and could be identified with MMP-8 specific chair-side test.

Deep periodontal lesions may be niches for non-periodontopathogenic micro-organisms with systemic effects like *C. pneumoniae* and possibly play a role in the transmission from one subject to another.

4 INTRODUCTION

Periodontal diseases are very common in all populations: nearly all dentate individuals suffer of gingivitis at some point of life. Also mild and moderate forms of chronic periodontitis are rather common (Sheiham 1997). However, severe forms of periodontitis with advanced tissue destruction (chronic periodontitis and aggressive forms of periodontitis) are rare worldwide (Albandar *et al.* 1999) as well as in European populations (Morris *et al.* 2001; Hugoson *et al.* 1995; Sheiham *et al.* 1986; Bourgeois *et al.* 1997).

Periodontal diagnosis is commonly based on clinical and radiological examination. With these methods it is possible to find out the current inflammatory status or already existing loss of periodontal attachment. Estimation of known risk factors such as plaque accumulation and oral hygiene status, smoking and some systemic factors such as diabetes give further information about the individual susceptibility which is not equal in all individuals. Only a proportion of individuals and tooth sites with gingival inflammation develop irreversible periodontal tissue loss (Albandar 2002). However, the commonly used methods are not optimal in expressing the current activity of the periodontal disease though non-existing bleeding on probing has been regarded as a reliable parameter of periodontal stability (Lang *et al.* 1986).

Periodontal tissue destruction is a consequence of the inflammatory reaction in a susceptible host initiated in response to bacterial colonisation forming a biofilm in the subgingival environment (McCulloch 1994a, b; Kinane 2000) and it is caused by host originating substances released by inflammatory cells and activated tissue cells.

During last years there has been a great interest in finding a biomarker which could reliably reveal active and tissue destroying periodontal disease, in order to identify those individuals who are at the risk of the transition of chronic gingivitis into irreversibly tissue destructing periodontitis or which could give us information about the periodontal treatment response. The main interest has focused on gingival crevicular fluid (GCF) as a potential diagnostic fluid but also saliva and oral rinses have been studied. GCF has a special role in site specific diagnosis because of its origin is very near the collecting site. Host derived enzymes and their

inhibitors, inflammatory and immune markers, tissue-breakdown products and enzymes of bacterial origin have been determined from GCF samples in numerous biomarker studies.

The ideal biomarker or combination of biomarkers should be an indicator of true tissue destruction, preferably in advance of the event. An easy to use chair-side diagnostic test for precise diagnostic purposes with good sensitivity and specificity would be ideal for clinical use. Enzymes of neutrophil origin are abundant in inflamed gingival pockets and may give information about ongoing destruction of periodontal attachment. These can be captured by means of GCF sampling for determination at a site specific level.

Human matrix metalloproteinases (MMPs) are a group of structurally related but genetically distinct endopeptidase enzymes that have defined roles in several physiologic but also in pathologic processes. They have for example the capacity to degrade practically all extracellular matrix and basement membrane matrices and components (Birkedal-Hansen *et al.* 1993; Sorsa *et al.* 2004a). Matrix metalloproteinase-8 (MMP-8) is the major collagenase enzyme present in inflamed human gingival tissue (Sorsa *et al.* 1988; Ingman *et al.* 1994a; McCulloch 1994a, b; Kiili *et al.* 2002). MMP-8 is also responsible for collagenase activity in chronic periodontitis GCF (Lee *et al.* 1995a; Ingman *et al.* 1996; Golub *et al.* 1997). MMP-8 is released from inflammatory cells in an inactive pro-form and is activated by different host- and microbe-derived factors at the site of inflammation. After periodontal treatment the amount of MMP-8 in GCF has been found to be reduced and mostly present in the latent form (Ingman *et al.* 1993). Thus MMP-8 is a potential biomarker of interest (Sorsa *et al.* 2004a, b).

The microbiota of the oral cavity and periodontal pockets is extremely complex. Periodontal pockets may serve as a niche for non-periodontopathogenic species and have a role as a possible reservoir for infective material in human to human contacts. *Chlamydia pneumoniae* has been shown to be most common of *Chlamydias* in humans causing harmless upper respiratory tract infections in systemically healthy individuals. Moreover, *C. pneumoniae* is known to cause 5—10% of cases of serious pneumonias and it is also the factor behind several diseases like obstructive pulmonary disease, asthma, atherosclerosis, reactive arthritis, and lung cancer (Saikku and Paavonen 2003). Although *C. pneumoniae* is not regarded as having a role in pathogenesis of periodontal diseases (Tran *et al.* 1997) it may contribute, for example by increasing the local host response process.

5 REVIEW OF THE LITERATURE

5.1. STRUCTURE OF HEALTHY PERIODONTIUM

Supporting tissues of the teeth, the periodontium, comprises: 1) the gingiva, 2) the periodontal ligament, 3) the root cementum, and 4) the alveolar bone which consists of the alveolar bone proper and the alveolar process which are continuous with each other. Normal gingival tissues form a protective barrier against infection and the frictional forces of mastication. In healthy periodontal tissues tissue degradation and repair are in balance and the periodontium maintains the structural and functional integrity.

Type I collagen is the major type of collagen in mature gingival connective tissue. Also type III collagen is localised in periodontal connective tissue while type IV collagen is the main collagenous component of the basement membrane (Chavrier *et al.* 1984). Type I collagen which is organised in dense bundles, maintains the integrity of the connective tissue as well as provides a major substratum for the attachment of the connective tissue cells.

The **gingiva** (attached, free marginal and interdental) may be regarded as a collar of masticatory mucosa around the teeth and it is attached to both teeth and alveolar processes. Structurally, the gingiva is composed of a densely collagenous lamina propria including the supra-alveolar fibres, blood and lymphatic vessels and nerves and two different kinds of epithelia: oral epithelium facing the oral cavity and sulcular epithelium facing the tooth (Schroeder and Listgarten 1997). On the contact surface between the tooth and the gingiva exists junctional epithelium (JE), a thin, non-keratinised epithelium with wide intercellular spaces which allow the inflammatory cells to migrate through the epithelium. The cells of the internal basal lamina of JE form the epithelial attachment to the tooth surface via hemidesmosomes. JE is a dynamic, constantly remodelling tissue with a high cellular turnover rate (Bosshardt and Lang 2005).

Periodontal ligament, a fibre apparatus connecting the tooth to the alveolar bone, is a unique tissue; small in proportion but definitively functionally important. It has similarities with other tendons and ligaments but it is specific because it inter-connects two different kind of oral hard tissue: instead of having two bone insertions it enters the radicular cementum at

the tooth end. The periodontal ligament is not only a suspensory complex but also detrimental for mechanotransdunction of signals from teeth to alveolar bone when physical forces are applied and when there is a requirement for rapid remodelling of bone (McCulloch *et al.* 2000). The extracellular matrix of periodontal ligament is a loose plexus of wavy collagen fibrils which immerse in a highly hydrated interfibrillar matrix. The major type of collagen in the periodontal ligament, type I, is embedded in type III collagen with a secondary role in the fibrous supportive structures (Embery 1990).

The main function of **cementum** is to attach the tooth to surrounding alveolar bone via the periodontal ligament. No physiologic resorption or remodelling occurs in cementum; instead a slow apposition of new cellular cementum continues throughout lifetime as a consequence of physiologic demands.

Alveolar bone is the least stable of periodontal structures which may be one factor in the pathogenesis of periodontitis. The organisation of alveolar bone is extremely complex, influenced by masticatory stress and other oral factors. The turnover rate is high because of the factors mentioned above but in the healthy/normal state resorption and formation of new bone is in balance and regulated by local and systemic factors.

5.2. DIFFERENT DISEASE STATES OF PERIODONTIUM

5.2.1. Classification of periodontal diseases

The latest classification of periodontal diseases is from the year 1999 (International Workshop for a Classification of Periodontal Diseases and Conditions 1999). It is mainly based on infection / host response paradigm (Armitage 2002), and all classification terminology dependent on age and rate of progression has been discarded (Armitage 1999). Categories I and II cover the most common periodontal diseases with the greatest prevalence: category I encompasses gingival diseases, and category II consists of chronic forms of periodontitis. Chronic periodontitis replaces the category adult periodontitis in the 1989 classification. Chronic periodontitis, which is common in adults, can also be seen in adolescents (Armitage 1999). However, term “chronic” does not mean “incurable” (Armitage 1999), and chronic periodontitis may be a constellation of diseases and not a single entity

(Armitage 2002). Aggressive periodontal diseases (localised and generalised) belong to category III which encompasses all highly destructive forms of periodontitis independent of the subject's age (in children, without any detectable underlying systemic disease). This category covers all forms of periodontitis which were in the 1989 classification under the category: early-onset periodontitis (prepubertal, juvenile and rapidly progressive periodontitis) (The American Academy of Periodontology 1989). Other categories of the 1999 classification are: periodontitis as a manifestation of systemic diseases (IV), necrotising periodontal diseases (V), abcesses of the periodontium (VI), periodontitis associated with endodontic lesions (VII), and developmental or acquired deformities and conditions (VIII).

The present-day classification is not based on etiology or pathology of periodontal diseases which would demand more knowledge about the host-microbial interactions and the environmental factors behind them. Instead it reflects the current understanding of the nature of periodontal diseases and should be modified when new knowledge becomes available (Armitage 2002).

5.2.2. Gingivitis

Almost 100% of the population suffers periodically from gingivitis, a reversible inflammation of the gingiva. The main etiological factor behind gingival inflammation is the accumulation of a biofilm on the tooth surface when oral hygiene measures are insufficient. With increasing bacterial load typical signs of inflammation appear: redness, change of the surface structure with oedema, and bleeding even when slightly touched with a tooth brush or periodontal probe. When bacterial plaque accumulates on the tooth surface, a neutrophil-dominated response starts, and in few days clinical signs of gingival inflammation can be seen with increased flow of gingival crevicular fluid (GCF). When causative factors (reasons for bacterial plaque accumulation, i.e. insufficient oral hygiene or iatrogenic factors) are eliminated, the gingival tissue returns to a healthy state (Løe *et al.* 1965). Though there is still no clinical means to recognise individual susceptibility for "dangerous" gingivitis with risk of progressing to periodontitis, there are known risk factors which should be considered when estimating the risk.

Even in a pristine state of periodontal health, an accumulation of neutrophil leucocytes is present in gingival connective tissue underneath the epithelium lining the gingival crevice. Inflammatory cells in the subepithelial portion of the lamina propria immediately under JE and inside it are part of normal homeostasis and an essential part of the defence system in this structure which is continuously challenged by microbes (Schroeder and Listgarten 1997).

One of the earliest changes associated with the transition from health to gingivitis is the increased GCF level of the neutrophil chemoattractant interleukin (IL) -8 (Tonetti *et al.* 1998). During this phase pocket epithelium forms epithelial pegs into the connective tissue, and the epithelial surface towards the connective tissue increases. Already, during early gingivitis, many collagen fibres in the gingiva are broken down. This makes space for extravasation of inflammatory cells and infiltration into the gingival connective tissue.

The inflammatory cell infiltrate of early gingivitis mainly consists of neutrophils. During the pathogenic process towards established gingivitis the infiltrate is characterised by the increase in plasma cells (10—30% of inflammatory cell infiltration), and plasma cells become the dominant cell (> 50%) in periodontitis i.e. when alveolar bone is lost and JE is migrating in an apical direction (Kinane and Lindhe 2003).

5.2.3. Chronic periodontitis

Untreated chronic gingivitis can stay unchanged for long periods, and epidemiological data suggests that only a proportion of individuals and tooth sites with gingival inflammation develop irreversible periodontal tissue loss, periodontitis (Albandar 2002). In epidemiological studies the worldwide prevalence of severe periodontal disease has been found to be low (Albandar *et al.* 1999). Advanced periodontal disease with deep pocketing is reported to be rare also in European populations (Morris *et al.* 2001; Hugoson *et al.* 1995; Sheiham *et al.* 1986; Bourgeois *et al.* 1997). In European subjects mild gingival inflammation is common as well as many adults have mild to moderate periodontal attachment loss at some sites of dentition (Sheiham 1997). A national survey in the USA showed that chronic periodontitis among dentate persons aged ≥ 30-years and with six or more remaining teeth is altogether common: 3.1% suffered from advanced, 9.5% from moderate, and 21.8% from mild chronic periodontitis (Albandar *et al.* 1999). In a recent

Finnish large scale population study (National Public Health Institute 2004) where periodontal disease was defined as having at least one periodontal pocket ≥ 4 mm, 70% of men and 60% of women over 44-years-old fulfilled this criterion. This means that gum diseases are common among Finnish adult population.

Typical for chronic periodontitis is a rather weak activity of subgingival bacteria, and host response where lymphocytes are the dominating cells. As a reaction to inflammation, normal periodontal tissue transforms to granulation tissue with large numbers of infiltrated plasma cells and attachment of the tooth is lost. Granulation tissue prevents invasion of micro-organisms and their virulence factors into the surrounding tissue and there exists a balance between the host and subgingival microbiota with minimal risk of systemic spread of infection. It is still unknown if chronic periodontitis progresses as bursts in individual sites or throughout the dentition at the same time or if the progression is gradual. All these forms may be possible at different time periods in the same dentition.

5.3. PATHOGENESIS OF PERIODONTITIS AND PERIODONTAL INFECTION

In periodontitis, the gram-negative microbial plaque colonises into the deepened gingival crevice and evokes a chronic inflammatory response. The inflammatory cascade starts with the penetration of lipopolysaccharides (LPS) from the surface of gram-negative bacteria into periodontal tissues. LPS stimulates monocytes/macrophages to secrete modulators of inflammation, like prostaglandin E_2 (PGE_2), IL-1, -6 and -8, tumour necrosis factor alpha (TNFα), and collagenases (MMPs), which in turn activate vascular smooth muscle cells, fibroblasts, further monocytes/macrophages, and osteoclasts to produce various proteolytic enzymes and stimulate bone resorption (**Figure 1**). This leads to clinical inflammation and periodontal attachment loss. The hallmark in the progression of gingivitis to periodontitis is regarded to be the conversion of JE to pocket epithelium. The clinical sign is the formation of periodontal pockets.

In addition to the role as a seal between the tooth and underlying tissues, JE participates in the host defence mechanisms and has an active role in the innate host defence (Bosshardt and Lang 2005). Periodontal pocket formation has been attributed to a loss of cell to cell contacts in the coronal portion of JE and the detachment of JE cells directly attached to the tooth

(DAT-cells) (Pöllänen *et al.* 2003). This focal disintegration of JE is further exacerbated by increased numbers of T- and B-lymphocytes, monocytes/macrophages and neutrophils (Schroeder and Listagarten 1997). Moreover, JE can be invaded by bacteria and bacterial products which can directly participate in the process of structural disintegration of JE (Bosshardt and Lang 2005).

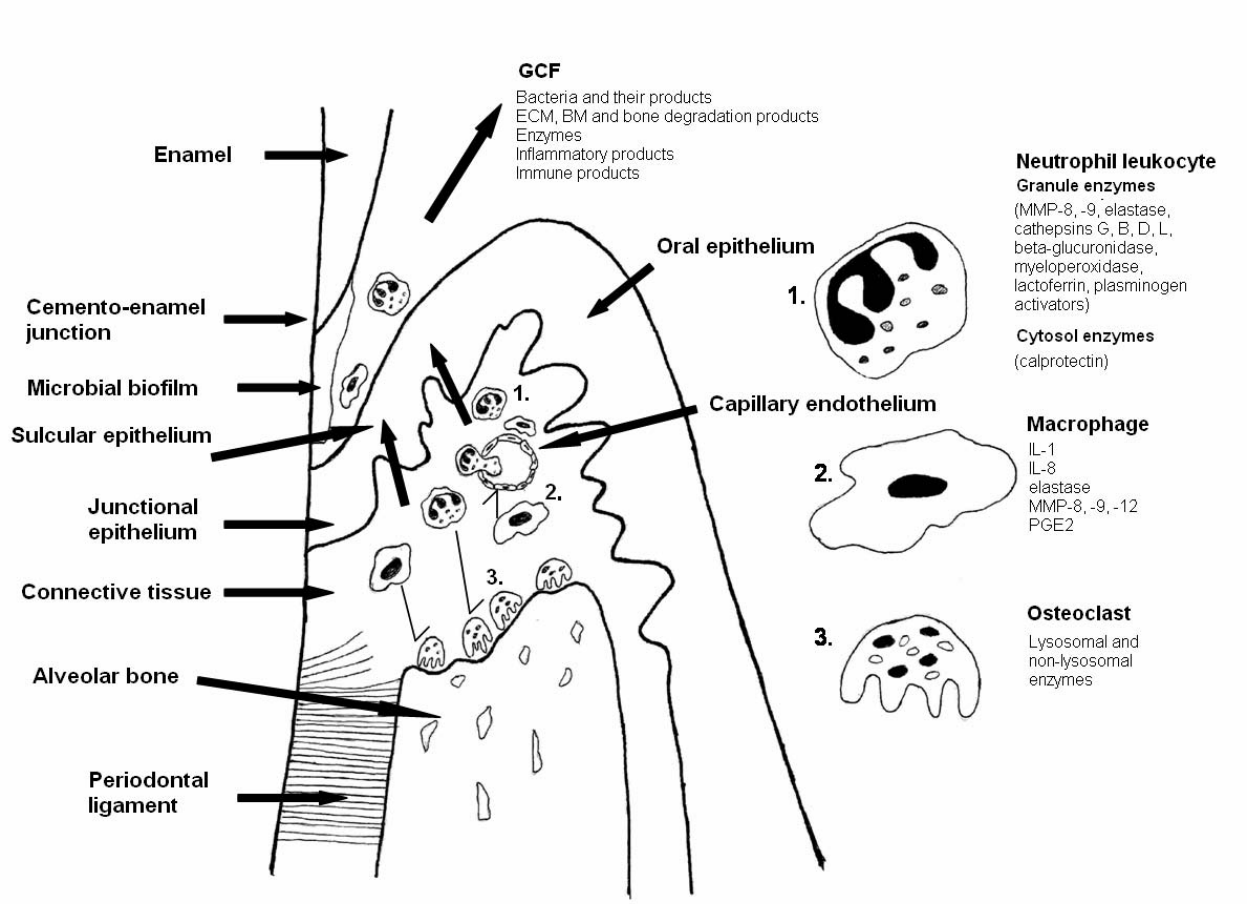

Figure 1. A microbial biofilm induces the host response. Activated macrophages induce vascular changes with increased vasopermeability. This leads to increased exudation and neutrophils are attracted to the site of inflammation. As a consequence of enzyme release by inflammatory cells, connective tissue as well as epithelial cells and basement membrane will be degraded (bystander damage). Osteoclasts will be activated to degrade alveolar bone. Some focal biomarkers of neutrophil and macrophage origin are shown.

JE cells express numerous cell adhesion molecules (CAMs). The expression of intercellular adhesion molecule-1 (ICAM-1) forms a gradient which is thought to be a mechanism guiding neutrophils towards the bottom of the sulcus to counteract the bacterial challenge (Tonetti 1997; Tonetti *et al.* 1998). JE expresses also IL-8, IL-1α, IL-1β, and TNFα especially in its coronal half (Miyauchi *et al.* 2001) and natural antibacterial peptides and proteins such as β-

defencins, LL-37 (human cathelidicin-derived peptide), and calprotectin in response to bacterial challenge (Dale 2002). The epithelium responds to bacteria also by signalling further host responses, by increasing proliferation, by changing differentiation and cell death, and it integrates innate and acquired immunity (Dale 2002).

A variety of factors are thought to contribute to the pathogenesis of periodontitis including inadequate oral hygiene, systemic diseases, genetic predisposition and smoking habits, all of which modify the host response as well as the composition of the oral microbiota (McCulloch 1994a, b; Kinane 2000). In a susceptible host periodontal tissue destruction is a consequence of the inflammatory reactions initiated in response to bacterial colonisation forming the biofilm in the subgingival environment (McCulloch 1994a, b; Kinane 2000).

Some gram-negative or opportunistic bacterial species identified in deep periodontal pockets by culture are regarded to be the primary causative microbial species for development of periodontitis. However, in all cases the relationship between periodontopathogenic cultivable bacterial species and periodontal disease is not unambiguous and the same species can be found from both diseased and healthy sites (Moore and Moore 1994; Socransky and Haffajee 1994). Current knowledge of the diversity of oral microbial species questions the role of the small number of pathogens as the sole cause of periodontal diseases (Slots 2005). Earlier adopted non-specific and specific disease models have been superseded by the ecological plaque hypothesis which implies that periodontitis is caused by resident flora which may contain pathogenic species (Marsh 1994) and that the relationship of certain bacteria in the pathogenesis of periodontal diseases is rather ecological than specific. The shift in the proportions of species may be a consequence of the changed gingival environment (Newman 1990). The complex periodontal microbiota differs in composition from site to site in the same subject and from subject to subject (Haffajee and Socransky 2005).

Molecular methods have revealed a tremendous diversity of oral and periodontal flora. The number of different micro-organisms recognised in the oral cavity has more than doubled in number by using molecular methods compared with the number isolated by culture techniques. This finding requires evaluating anew which species participate in the pathogenesis of different oral diseases (Tanner and Izard 2005). Tanner and Izard (2005) suggested that the detection of disease-associated mediators of host tissues should be used in association with detecting selected species for the assessment of microbial pathogenity.

The possible role of viruses in the etiology and pathogenesis of periodontal diseases has recently been speculated and herpes viruses have been studied as putative periodontopathogens (Slots 2005). An apparent association between human cytomegalovirus and progressing periodontitis has been detected (Kamma *et al.* 2001a; Kubar *et al.* 2004). Periodontal inflammatory cells contain nucleic acid sequences originating from herpes viruses and periodontal inflammatory cells infected by herpes viruses can express increased levels of tissue-damaging cytokines and chemokines (Mogensen and Paludan 2001).

The main immune defences against subgingival infection are neutrophil leukocytes (polymorphonuclear leukocytes, PMN), antibodies and complement, which work in concert. Antibodies and complement work synergistically in opsonising bacteria, and chemotaxis and phagocytosis by neutrophils becomes more effective. The abundance of released enzymes from neutrophils, other inflammatory cells and activated tissue cells cause damage to host tissue also i.e. bystander damage.

5.3.1. The periodontal pocket as a niche for *Chlamydia pneumoniae*

The oral microbiota consists of 500—600 species most of which have not been associated with the pathogenesis of periodontitis. However, periodontal pockets may harbour species which may be relevant for systemic health.

Chlamydias are tiny gram-negative bacteria of which *Chlamydia pneumoniae* has been identified as a separate species in 1989. *C. pneumoniae* has been shown to be the most common *Chlamydia* in humans causing several diseases like obstructive pulmonary disease, asthma, atherosclerosis, reactive arthritis, and lung cancer (Saikku and Paavonen 2003). Nearly all individuals suffer from *C. pneumoniae* infection 2—3 times during their lifetime but in most cases the infection is a mild upper respiratory tract infection. However, *C. pneumoniae* is the factor behind 5—10% of cases of serious pneumonia which can be fierce and lead to death in patients with poor resistance (Saikku and Paavonen 2003). There is evidence that *C. pneumoniae* may play a role in chronic lung diseases as well as in atherosclerosis plaque formation (Mosorin *et al*. 2000; Corrado *et al.* 2005; Volanen *et al.* 2006), in acute heart attack (Arcari *et al.* 2005), and in cardiac valvular disorders (Nyström-Rosander *et al.* 2003; Skowasch *et al.* 2003). Chronic inflammation caused by *C.*

pneumoniae may even be the causative agent behind these disorders (Saikku and Paavonen 2003).

C. pneumoniae has been identified also in the oropharynx: during epidemics *Chlamydia* species have been found to be responsible for up to 9% of cases of pharyngitis in adults (Huovinen *et al*. 1989). *C. pneumoniae* is not regarded to have a role in the pathogenesis of periodontitis but deep periodontal pockets may be a niche for micro-organisms which are not regarded as periodontopathogenic bacteria such as *C. pneumoniae*. Thus periodontal pockets could be the reservoir of *C. pneumoniae* in otherwise symptomless carriers and a source of infection through transmission to more susceptible individuals. Tran *et al.* (1997), using a 16S rRNA-based identification method, did not find *C. pneumoniae* in subgingival dental plaque samples from 50 adult patients with advanced periodontal disease thus concluding that *C. pneumoniae* is not part of the periodontopathogenic microbiota in humans. Interestingly, *C. pneumoniae* infection has lately been found with a nested polymerase chain reaction designed to identify *C. pneumoniae* DNA to be highly prevalent in cyclosporine-induced post-transplant gingival overgrowth (Worm *et al.* 2004).

5.4. SMOKING AS AN ETIOLOGICAL RISK FACTOR FOR PERIODONTITIS

Smoking is a risk factor for the incidence and progression of periodontal disease (Bergström and Preber 1994; Grossi *et al*. 1994). From the data of a national survey in the USA it was estimated that in US adults with periodontitis, 41.9% cases were attributable to current smoking and 10.9% to former smoking (Tomar and Asma 2000). There is strong evidence suggesting an association between intensity and duration of smoking and periodontal attachment loss and severity of periodontitis (Grossi *et al.* 1995, 1997; Martinez-Canut *et al*. 1995; Gonzales *et al.* 1996; Bergström *et al.* 2000). Smoking of one, 2—10 or 11—20 cigarettes per day was associated with an increase in the prevalence of attachment loss of 0.5%, 5% and 10%, respectively in a study of 889 periodontal patients (Martinez-Canut *et al.* 1995). The number of smoking years has been shown to be associated with tooth loss and periodontitis irrespective of other environmental and behavioural factors (Jette *et al.* 1993) and smoking status is independently significantly related to the attachment level of teeth (Bergström *et al.* 2000; Bergström 2004). The effect is additive with increasing age (Haffajee and Socransky 2001a). However, there are also contradicting findings where smokers do not

have greater progression of periodontal disease as measured by pocket probing depth (PD) or attachment loss (AL) (Chen *et al.* 2001).

Smoking impairs the normal defence mechanisms of the host response (Quinn *et al.* 1998; Mooney *et al.* 2001) and stimulates destructive effects of the host response (Gustafsson *et al.* 2000; Persson *et al.* 2001). However, neither salivary nor GCF cotinine levels have been found to correlate significantly with PD, AL or tooth loss in smokers though mean GCF cotinine was about four times higher than mean salivary cotinine levels (Chen *et al.* 2001).

The effect of smoking on periodontal health seems to be unrelated to the composition of the subgingival microflora (Preber *et al.* 1992; Stoltenberg *et al*. 1993; Darby *et al.* 2000; Boström *et al.* 2001). However, a difference between smoker (S) and non-smoker (NS) subjects' subgingival microbiota has been found in the prevalence of species rather than counts or proportions of organisms (Haffajee and Socransky 2001b). There is also a finding with no statistical difference, in the occurrence of periodontopathogens (*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*) (Boström *et al.* 1998a). In smokers no microbial species is specifically associated with bleeding on probing (BOP) (Boström *et al*. 2001).

The typical feature of smokers' gingival tissue is keratinisation which may mask inflammation. Less BOP among smokers has been confirmed in several studies (Alavi *et al*. 1995; Boström *et al*. 2001; Bergström and Boström 2001; Chen *et al*. 2001; Erdemir *et al.* 2004) even in spite of a 2- to 3-fold greater disease severity measured as the number of deep periodontal pockets (Bergström and Boström 2001). The mechanism in smokers may be related rather to inflammation events than to vascular functions *per se* because periodontally healthy smokers and non-smokers do not differ regarding hemorrhagic responsiveness or vascular density (Bergström and Boström 2001). The effect of nicotine seems to be directed to vascular dynamics and cellular metabolism and it is not only due to vasoconstrictive action (Bergström and Boström 2001). In an experimental gingivitis study in healthy S and NS subjects Giannopoulou *et al.* (2003a) noted after a 10 day period of plaque accumulation significantly lower gingival index (GI) and less BOP in S than in NS subjects.

There are also different or contradictory results where smokers demonstrated significantly more bleeding on probing (Renvert *et al.* 1998). Müller *et al.* (2002) observed in their

longitudinal study that smokers' gingiva tended to convert to bleeding irrespective of presence of known risk factors for gingivitis in a gingivitis study investigating healthy young adults. However, in another study vasculature of gingival tissues between periodontally healthy smokers and non-smokers was not different (Persson and Bergström 1998).

There is a general opinion that response to periodontal procedures (Preber and Bergström 1990; Renvert *et al.* 1998) and the result of periodontal surgery (Boström *et al.* 1998a) are less favourable among smokers. Smoking affects adversely on the long-term prognosis of periodontal disease. Smokers are twice as likely to loose teeth compared with non-smokers during the maintenance phase (McGuire and Nunn 1996) and up to 90% of refractory periodontitis patients are smokers (MacFarlane *et al.* 1992; Magnusson *et al.* 1994; Magnusson and Walker 1996).

Several studies have demonstrated that the effects of smoking are somewhat reversible, and smoking cessation markedly improves the prognosis and outcome of periodontal treatment (Grossi *et al.* 1997; Kaldahl *et al.* 1996; Scabbia *et al.* 2001). In comparing former smokers with current smokers the prevalence and severity of periodontitis is less (Haber 1994), the rate of tooth loss is reduced (Krall *et al.* 1997) and the progression of bone loss is retarded (Bolin *et al.* 1993) among former smokers.

5.5. COMMONLY USED METHODS FOR DIAGNOSING PERIODONTITIS

5.5.1. Clinical and radiographic diagnosis

Periodontal diseases are quiescent in most cases with minimal clinical signs. Changes are often severe before the diagnosis is made. About 8—10% of individuals can develop extensive tissue destruction leading to loss of teeth (Papapanou 1999). Clinically, signs of inflammation (changed colour and consistency of gingiva, bleeding on probing and suppuration) and changes in PD and clinical attachment level (CAL) measured with periodontal probe can be observed along with radiological findings. PD and CAL as well as radiological findings can tell us about destruction that has already happened. No relevant information about the current activity of periodontitis can be achieved with these measures. Inflammation is an important finding in periodontal examination. However, other clinical

indices except PD fail to predict change in CAL (Adonogianaki *et al.* 1996). Non-existing BOP has been regarded as the golden standard when clinically estimating periodontal health status even in deep periodontal pockets. A bleeding site is at 30% risk for loss of attachment (Lang *et al.* 1986), which means that single registration of BOP is of minimal value in predicting disease progression (low sensitivity). Lang *et al.* (1986) regarded the non-existing BOP as a good guarantee of non-existing disease (high specificity). Plaque scores are a poor predictor of periodontal disease progression (Haffajee *et al.* 1983; Kaldahl *et al.* 1990), but patients maintaining a good standard of oral hygiene are less in danger of disease recurrence or progression even with teeth where the initial prognosis is questionable or hopeless (Chase and Low 1993; McGuire 1991).

Deep periodontal pockets are at constant risk of disease progression; depth of gingival pockets correlates with future loss of attachment (Claffey and Egelberg 1995; Nieminen 1997). Individuals with deep periodontal pockets and attachment loss with several bleeding sites are at a more severe risk of disease progression than individuals with minimal BOP (Claffey *et al.* 1990). However, there are also reports that the residual probing depth is to a lesser extent than BOP a predictor of disease activity (Armitage 1996).

5.5.2. Microbiological diagnosis

Only a few of several hundred recognized species of micro-organisms inhabiting the gingival crevice are regarded to play roles in the etiology of periodontal diseases. These few species are associated with the initiation and progression of periodontal disease (Socransky and Haffajee 1991, 1992). However, the presence of putative periodontopathogens in the gingival crevice is not enough to cause periodontal disease: a critical mass of bacteria is needed to cause tissue damaging immunomodulatory effects in the susceptible host.

Subgingival plaque can be collected with curettes or by adsorbing subgingival plaque onto endodontic paper points for microbiological analysis. With a curette plaque can be collected from the entire pocket, while a paper point adsorbs mostly the outer layer of the biofilm (Socransky and Haffajee 2002). Each periodontal pocket has a unique microflora, and the sample from a few sites only is necessarily not representative of other diseased sites. Methods to analyze plaque samples include microscopy, culture techniques, enzymatic

assays, immunoassays, nucleic acid probes, and PCR assays. Tests available for the clinician can detect only some bacterial and fungal species that have been documented to have an etiologic role in periodontal diseases. Microbial culturing on selective or non-selective media, though capable of identifying only some bacterial species, has the advantage of assessing antibiotic sensitivity. However, low levels of micro-organisms in the sample can not be detected.

The sample can be analyzed immediately by microscopy to find out the relative proportions of coccal and filamentous-shaped organisms (Offenbacher *et al.* 1985; Rams 1986). The main use of microscopy is to observe the shift in the composition of the flora with periodontal therapy but has not been shown to predict the recurrence of the disease (Listgarten *et al.* 1986).

Immunoassays such as immunofluorescense microscopy, enzyme-linked immunosorbent assay (ELISA), membrane assays and latex agglutination assays, and nucleic acid probes as well as PCR assays have been used for scientific rather than for clinical purposes. These methods have higher sensitivity than culture methods but do not permit the evaluation of the antibiotic sensitivity of the flora. Some PCR assays can detect virtually all micro-organisms in a plaque sample (Zambon and Haraszthy 1995) but micro-organisms which are present in plaque in very small quantities may not necessarily contribute to the development and progression of periodontal disease. However, findings suggest that neither culture nor molecular methods alone can reliably evaluate the oral microbiota and different approaches are needed (Tanner and Izard 2005).

Routine testing of periodontopathogens in chronic periodontitis offers limited benefit (Jeffcoat *et al.* 1997). Though bacteria related to periodontal diseases have been studied in several investigations, many of these are not valuable if we wish to estimate evidence. A systematic review could not find bacterial species or their combinations which could differentiate individuals with aggressive periodontitis from those with stable one (Mombelli *et al.* 2002). Moreover, the results of plaque analysis do not give any information regarding the risk of disease progression. Microbial diagnosis can be of value, however, in treatment planning of periodontitis patients with a poor treatment outcome to meticulous conventional mechanical hygiene phase periodontal therapy (SRP), patients suffering from aggressive periodontitis or who are medically compromised.

5.6. MATRIX METALLOPROTEINASES (MMPs) IN THE PATHOGENESIS OF PERIODONTITIS

Human matrix metalloproteinases (MMPs) are a group of at least 23 structurally related but genetically distinct endopeptidase enzymes that cleave the internal peptide bonds of proteins (Kähäri and Saarialho-Kere 1999). They have the capacity to degrade practically all extracellular matrix (ECM) and basement membrane (BM) matrices and components (Birkedal-Hansen *et al.* 1993; Sorsa *et al.* 2004a) and inactivate and process serpins, growth factors, cytokines, cell adhesion molecules, apoptosis factors, and chemokines (Sternlich and Werb 2001). In healthy subjects MMPs, along with other extracellular proteinases are involved in diverse physiological events such as tissue remodelling, coagulation and fibrinolysis, apoptosis, wound repair, bone remodelling, host defence and immunomodulatory reactions, regulation of inflammatory responses, reproduction, fertilisation, and development. In disease, MMPs are associated with several inflammatory conditions involving tissue injury, such as lung, skin and eye diseases as well as diseases of skeletal system, and cancer (Owen and Campbell 1999a; Sternlich and Werb 2001; Nyberg *et al.* 2006). MMPs are neutral proteinases and consequently functionally active and catalytically competent at physiological pH and temperature (Nyberg *et al.* 2006).

MMPs are 32—49% homologous at the amino acid level, and most MMPs share a common basic five domain prototype structure (**Figure 2**) (Murphy and Docherty 1992; Nagase *et al.* 2005). A typical MMP consists of propeptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide which is also called the hinge region of variable length, and a hemopexin domain of about 200 amino acids (Nagase *et al.* 2005). MMPs are grouped mainly based on substrate specificity and molecular domain structure to **1)** interstitial collagenases (MMP-1, -8 and -13), **2)** gelatinases (type IV collagenases; MMP-2 and -9), **3)** stromelysins (MMP-3, -10 and -11), **4)** matrilysins (MMP-7, -26), **5)** membrane-type (MT-)MMPs (transmembrane type and GPI-anchored elastase) and **6)** other MMPs including metalloelastase (macrophage elastase, MMP-12), enamelysin (MMP-20), epilysin (MMP-28), CA-MMP (MMP-23), and MMP-19, -21, and -27 (Nagase *et*

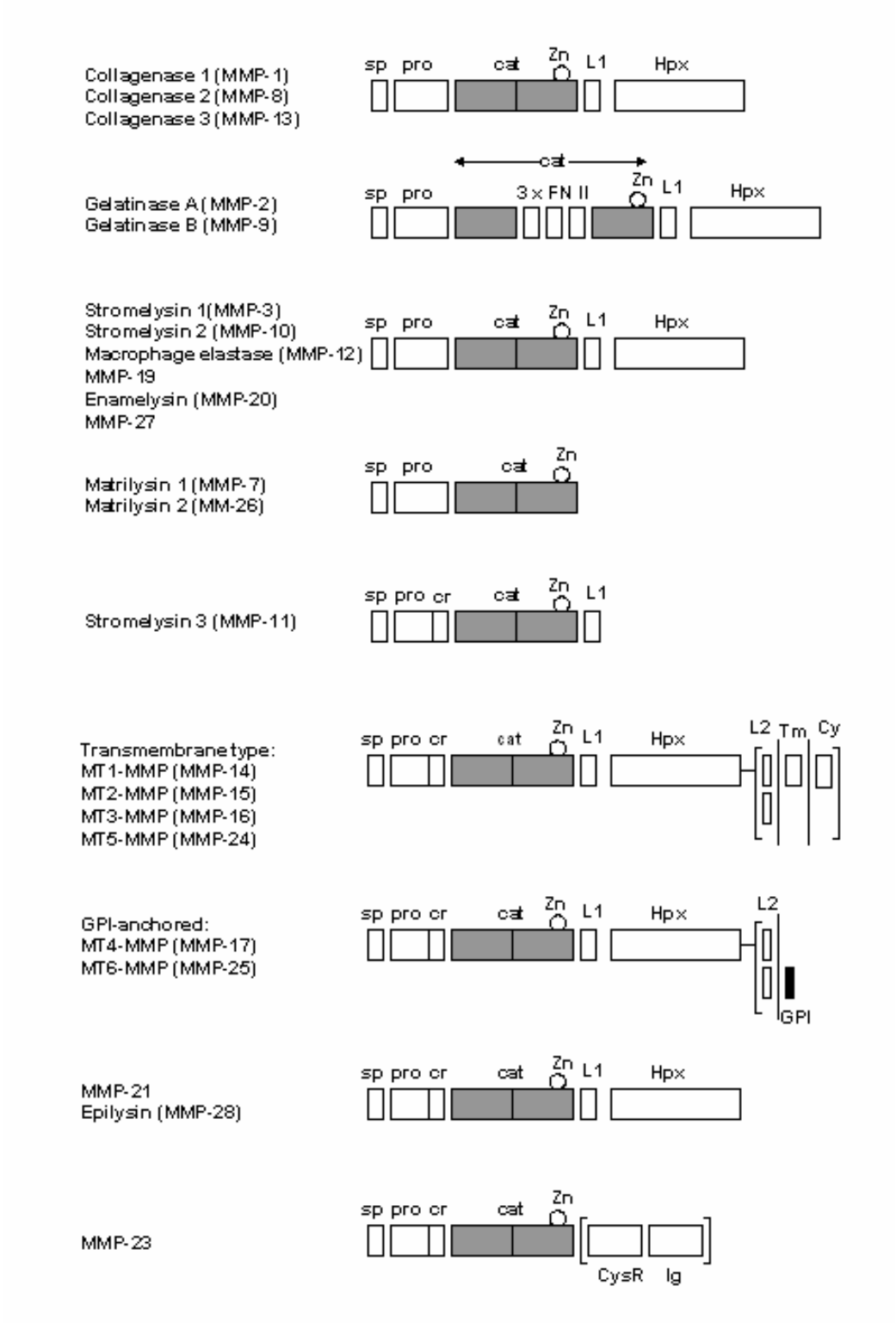

Figure 2. Matrix metalloproteinases and their domain structures. Sp, signal peptide; pro, pro-domain; cr, proprotein convertase recognition center; cat, catalytic domain; Zn, zinc; FN II, fibronectin type II motif; Hpx, hemopexin domain; L, linker; Tm, transmembrane domain; GPI, glycosylphosphatidylinositol anchor; Cy, cytoplasmic tail; CysR, cysteine rich domain; Ig, immunoglobulin domain. (Modified from Nagase *et al.* 2005 and Sternlich and Werb 2001.)

al. 2005). MMPs are catalytically dependent on intrinsic Zn^{2+} ions and extrinsic Ca^{2+} for full and optimal activity (Stöcker *et al.* 1995).

All MMPs are synthesised as inactive proenzymes or zymogens, and their latency is maintained by an unpaired cysteine sulfhydryl group near the C-terminal end of the propeptide prodomain (Van Wart and Birkedal-Hansen 1990). Thirteen MMPs are secreted from the cell as proMMPs and ten MMPs which possess a furin-like proprotein convertase recognition center at the end of the propeptide, are likely to be activated intracellularly. These latter mentioned MMPs are secreted (MMP-11, -21, -23, -28) or cell surface bound (MT-MMPs) as active enzymes (Nagase *et al.* 2005). Activation is result of at least partial removal of the prodomain into a lower molecular weight active form through various pathways, such as stepwise or direct extra-cellular activation, activation on the cell surface or intracellular activation (Nagase 1997). *In vivo* MMPs have been suggested to be activated alone or in concert with reactive oxygen species and human and bacterial proteinases (Saari *et al*. 1990; Sorsa *et al.* 1992a, 1997; Moilanen *et al.* 2003; Nyberg *et al*. 2006). MMPs have somewhat overlapping functions probably as a safeguard against loss of regulatory control, which confounds the efforts to understand function of MMPs *in vivo* (Sternlich and Werb 2001).

ECM turnover is regulated by a delicate equilibrium between the production, activation, and inhibition of proteolytic enzymes. The balance between expression and synthesis of MMPs is regulated by their major endogenous inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) which are synthesised by connective tissue cells and leucocytes and form non-covalent complexes with MMPs (Murphy *et al.* 1989). Currently four TIMPs are known with different substrate specificities (Brew *et al.* 2000). TIMP-1 and -2 are soluble inhibitors that are present in numerous body fluids (Moses 1997), and TIMP-3 is an insoluble inhibitor bound to ECM heparin sulfate proteoglycans and especially expressed in the eye (Langton *et al.* 1998). In addition to TIMPs, MMP activity and activation can be controlled in other ways such as proteolytic degradation and inactivation, with chelating agents like EDTA, and by non-specific endogenous inhibitor α2-macroglobulin (α2-MG) which is an abundant plasma protein and the major inhibitor of MMPs in tissue fluids whereas TIMPs act locally (Sternlich and Werb 2001).

MMPs influence ECM regulation by altering the composition and structural organization of the ECM, thereby altering matrix-derived signals. This proteolytic ECM remodelling results in the release of other modular break-down products with biologic activity (Sternlich and Werb 2001; Nyberg *et al.* 2003). Most cells must adhere to natural or provisional matrices to survive, thus MMP-mediated disruption of subcellular matrices can induce apoptosis in anchorage-dependent cells and plays an important role in normal physiologic cell death (Sternlich and Werb 2001).

Gingival tissue extracts, GCF and other samples have shown the type I collagen cleavage pattern which is a characteristic of mammalian collagenases [cleaving of native collagen at a single locus, resulting in formation of two fragments, (αA) 3/4 and (αB) 1/4 (Mancini *et al.* 1999; Uitto *et al.* 2003; Sorsa *et al.* 2004a; Safkan-Seppälä *et al.* 2006)], not of bacterial collagenases [attack at multiple sites, producing many short peptide fragments (Sorsa *et al.* 1987)]. MMPs are regarded as key mediators in tissue destruction in periodontitis (McCulloch 1994a, b; Kinane 2000; Sorsa *et al.* 2004a, b), and there is evidence that irreversible connective-tissue degradation in periodontitis results from an imbalance between MMPs and their locally acting tissue inhibitors TIMPs (Ingman *et al.* 1996; Sorsa *et al.* 2004a, b) and collagen degradation of connective tissue and the organic component of bone in inflamed periodontal tissues are likely to occur via MMPs (Sodek and Overall 1988; Birkedal-Hansen 1993) which eventually act as a cascade (Tervahartiala *et al.* 2000; Beklen *et al.* 2006).

5.6.1. Role of MMP-8 (collagenase-2) in periodontal connective tissue degradation

Among the MMPs, collagenases (MMP-1, -8 and -13) are the most substrate specific regarding their unique capacity to degrade native interstitial collagens. The hemopexin domain in their structure is essential for collagenases to cleave the triple helix of collagen (Nagase *et al.* 2005). They can also degrade other matrix macromolecules and inactivate serpins as well as modify pro- and anti-inflammatory mediators (Owen and Campbell 1999b; Sorsa *et al.* 2004a; Ala-aho and Kähäri 2005).

MMP-8 (**collagenase-2**, neutrophil collagenase) is synthesised by differentiating granulocytes in the bone marrow and stored in specific granules of circulating neutrophils.

However, there are also other cellular sources of MMP-8 such as sulcular epithelial cells, gingival and periodontal ligament fibroblasts, monocytes/macrophages, and plasma cells (Hanemaaijer *et al.* 1997; Tervahartiala *et al.* 2000; Wahlgren *et al.* 2001; Prikk *et al.* 2001, 2002; Sorsa *et al.* 2004a, b) thus indicating that sulcular epithelial and basal cells can interact with the tissue destructive MMP network (Kornman *et al.* 1997a; Tervahartiala 2003). There is also evidence that human cytokine-stimulated synovial and gingival fibroblasts, monocyte/macrophages, endothelial cells and joint chondrocytes can express mesenchymal-type MMP-8 *de novo* indicating that the regulation of tissue destruction by MMP-8 is more complicated than previously known (Weiss 1989; Hanemaaijer *et al.* 1997; Kiili *et al.* 2002; Sorsa *et al.* 2004a, b; Cox *et al.* 2006).

MMP-8 is released from neutrophils in a latent, inactive proform and becomes activated during periodontal inflammation by independent and/or combined actions of host-derived inflammatory mediators, such as TNFα and IL-1β, and microbial-derived proteases and reactive oxygen species (ROS) produced by triggered neutrophils (Weiss 1989; Ryan *et al.* 1996; Sorsa *et al.* 1992a, 2004a). The molecular mass of MMP-8 varies in different publications between 50 and 85 kDa, and forms as small as 20 kDa have been reported (Seltzer *et al.* 1981; Hasty *et al.* 1986; Sorsa *et al.* 1994) reflecting different degrees of MMP-8 glycosylation and/or whether the enzyme is in latent or activated/truncated form. Naturally activated MMP-8 obtained from peripheral neutrophils can be detected by immunoblotting at 65—70 kDa, and the MMP-8 in GCF migrates primarily as a 60 kDa form with smaller amounts of 78 kDa species, corresponding to active and latent forms of the enzyme, respectively (Ding *et al.* 1996, 1997; Romanelli *et al.* 1999). MMP-8 activity can be blocked with TIMP-1 and -2 as well as with tetracyclines (doxycycline), chemically modified non-antimicrobial tetracycline-derivatives, bisphosphonates and EDTA (Sorsa *et al.* 1994; Greenwald *et al.* 1998; Mancini *et al.* 1999; Teronen *et al.* 1999; Sorsa and Golub 2005).

The known ECM substrates of MMP-8 are collagens I—III, VII, X, gelatine, proteoglycans, bradykinin, angiotensin 1, fibrinogen, substance P, and aggrecan as well as pro- and anti-inflammatory cytokines/mediators, and MMP-8 can inactivate α2-MG and α1-proteinase inhibitor (Owen and Campbell 1999b; Sternlicht and Werb 2001). MMP-8 has been shown to play an important role in the periodontal tissue destruction. Especially it is the main collagenase in chronic periodontitis (Tervahartiala *et al.* 2000; Sorsa *et al.* 2004a, b). MMP-8 activity is associated also with inflammation related to gingivitis. In gingivitis slightly

elevated MMP-8 levels can be detected in GCF but as the latent, inactive proform (Sorsa *et al.* 1988; Lee *et al.* 1995a).

In chronic periodontitis MMP-8 represents 90—95% of GCF collagenase activity (Lee *et al.* 1995a; Ingman *et al.* 1996; Golub *et al.* 1997). During the active, progressing phase of periodontitis, MMP-8 levels in GCF are significantly elevated, and the MMP-8 will be almost completely converted to the active form (McCulloch 1994a, b; Sorsa *et al.* 1988; Lee *et al.* 1995a; Ingman *et al.* 1996). Higher levels of collagenase activity have been detected in the GCF of patients with progressive loss of periodontal connective tissue attachment compared to periodontitis patients whose condition is stable, and gingivitis patients (Lee *et al.* 1995a; Romanelli *et al.* 1999; Mancini *et al.* 1999; Pozo *et al.* 2005). A significant decrease in GCF MMP-8 activity following successful periodontal treatment has been shown (Lee *et al.* 1995a; Kiili *et al.* 2002; Figueredo *et al.* 2004) and after periodontal treatment more collagenase was found to be present in the latent form (Ingman *et al.* 1993).

Also high levels of active collagenase in salivary and mouthrinse samples seem to reflect the periodontal disease status because high levels of collagenase activity have been demonstrated both in saliva of patients with untreated chronic periodontitis and localised early-onset or aggressive periodontitis (Gangbar *et al.* 1990; Uitto *et al.* 1990; Ingman *et al.* 1993).

MMP-8 is also the major collagenase present in inflamed human gingival tissue (Sorsa *et al.* 1988; Ingman *et al.* 1994a; McCulloch 1994a, b; Kiili *et al.* 2002). Extracts of periodontitis patients' untreated gingival tissue in contrast to healthy subjects' gingiva contain pathologically elevated levels of MMP-8 in a catalytically active form (Sorsa *et al.* 1988). MMP-8 is also the major MMP present in human mature dental plaque (Sorsa *et al*. 1995).

Increased MMP-8 levels have been detected also in other inflammatory diseases such as rheumatoid arthritis, chronic lung inflammations and eye diseases (Konttinen *et al.* 1991; Prikk *et al.* 2001, 2002; Holopainen *et al.* 2003; Määttä *et al.* 2005, 2006a). Recently MMP-8 has been shown to exert unexpected anti-inflammatory or protective characteristics in LPS-induced lung inflammation and allergen-induced granulocytic lung inflammation probably by regulating inflammatory cell apoptosis and by processing anti-inflammatory cytokines and chemokines (Owen *et al*. 2004; Sorsa *et al.* 2004a; Gueders *et al.* 2005).

5.6.2. Other collagenases

Fibroblast-type collagenase **MMP-1** (**collagenase-1**) is transcribed and expressed mainly by human fibroblasts but also by keratinocytes, endothelial cells, monocytes/macrophages, osteoblasts, chondrocytes as well as malignant tumour cells (Birkedal-Hansen 1993). MMP-1 is produced as glycosylated proforms of 52 kDa and 57 kDa in size, and as activated species the sizes are 42 kDa and 47 kDa, respectively. MMP-1 is also found intracellularly and it may act on intracellular proteins (Nagase *et al.* 2005). MMP-1 is effective in hydrolysing type III collagen, but it is less efficient enzyme than MMP-8 in degrading ECM components and it is inhibited by TIMP-1. MMP-1 is regarded to be more associated with normal tissue remodelling than with tissue degrading processes (Birkedal-Hansen 1993; Ingman *et al.* 1994a, Golub *et al.* 1998).

MMP-1 seems to be associated rather with localised juvenile periodontitis (LJP) than with chronic (adult) periodontitis (Suomalainen *et al.* 1991; Sorsa *et al.* 1992b). Ingman *et al.* (1996) detected elevated levels of MMP-1 from LJP patients' GCF compared with adult periodontitis (AP) patients' or healthy controls' GCF. Low levels of MMP-1 have been found to be present in AP GCF (Ingman *et al.* 1996). This is in concert with the finding reported by Haerian *et al.* (1995) who detected MMP-1 in only 20.8% of all studied periodontitis sites, and the levels did not correlate with the periodontal disease status. On the other hand Tüter *et al.* (2002) found elevated levels of MMP-1 in chronic periodontitis patients' GCF compared to periodontally healthy persons, and the levels decreased with hygiene phase periodontal treatment. Results from the study made with gingival biopsies from inflamed and healthy periodontal tissue also suggested that MMP-1 rather than MMP-8 may play an important role in the initiation of collagen degradation in periodontal disease but possibly MMP-8 plays an important role in periodontal tissue destruction (Aiba *et al.* 1996).

Epithelial cells may respond strongly to exogenous factors and produce a variety of bioactive molecules such as various cytokines and proteolytic enzymes, like **MMP-13** (**collagenase-3**). Expression of MMP-13, first identified in breast carcinomas (Freije *et al.* 1994), has been reported in the pocket epithelium in human periodontitis helping the pocket epithelium to invade into periodontal connective tissue (Uitto *et al.* 1998; Tervahartiala *et al.* 2000; Kiili *et al.* 2002), and in macrophage-like cells (Kiili *et al.* 2002) and in fibroblasts. MMP-13

degrades type II collagen ten-fold more efficiently than other collagenases (Knäuper *et al.* 1996), and it cleaves gelatin efficiently (Lindy *et al.* 1997). Secreted as 60 kDa glycoprotein, it is cleaved to 48 kDa form during proteolytic activation (Moilanen *et al.* 2003).

Immunoblots for MMP-13 in periodontitis patients' pre-treatment GCF samples showed complex, proenzyme and activated enzyme forms, and following SRP there were significant reductions in band absorbance together with clinical parameters (Kiili *et al.* 2002). This finding confirmed results of a study where GCF from periodontitis patients showed active, proenzyme and fragmented forms of MMP-13 (Golub *et al.* 1997; Ilgenli *et al.* 2006). Ejeil *et al.* (2003) detected decrease in collagen fibres in the presence of MMP-13 with MMPs -2, -9 and -1 in human gingival explants. They concluded that these metalloproteinases are involved in ECM degradation in periodontitis. Cox *et al.* (2006) showed that gingival fibroblasts from chronic adult periodontitis patients cultured in contact with type I collagen and stimulated by IL-1β, secreted and activated MMP-13 together with other collagenolytic MMPs (MMP-1, -2, -8 and -14). However, Mancini *et al.* (1999) could not detect MMP-13 in periodontitis patients' mouthrinse samples by collagenase activity assays or Western blots.

5.6.3. Gelatinases

Also **gelatinases MMP-2** and **MMP-9** are suggested to participate in periodontal tissue destruction because significantly higher levels of MMP-9 and elevated levels of MMP-2 have been found in periodontitis patients' GCF and the levels decreased with conventional periodontal treatment (Mäkelä *et al.* 1994; Ingman *et al*. 1996; Westerlund *et al.* 1996; Beklen *et al.* 2006). Gelatinases further degrade gelatins (collagens that have been denatured by interstitial collagenases) as well as collagens IV, V, VII, X and XI, elastin, and BM components (Owen and Campbell 1999b; Beklen *et al.* 2006). Both gelatinases have in their structure three repeats of a fibronectin type II motif in the catalytic domain which are necessary to effectively cleave type IV collagen, elastin, and gelatins (Nagase *et al.* 2005).

72 kDa gelatinase A, MMP-2, is produced predominantly by fibroblasts and other connective tissue cells. MMP-2 degrades preferentially elastin. Like MMP-1, MMP-2 can act also on intracellular proteins (Nagase *et al.* 2005). MMP-2 has collagenolytic activity, weaker than MMP-1 in solution, but it is recruited to the cell surface and is activated by membrane-bound

MT-MMPs and may thus express collagenolytic activity near the cell (Nagase *et al*. 2005). Increased levels of MMP-2 have been detected in chronic periodontitis-affected gingival tissue localised in close vicinity to the sulcular basement membrane region (Korostoff *et al.* 2000; Pirilä *et al.* 2001).

MMP-9 (gelatinase B) degrades more effectively gelatine as well as collagens types I, II, III, V, VI and X, fibronectin, fibrillin, and aggrecan (Konttinen *et al.* 1998; Kähäri and Saarialho-Kere 1999). In periodontitis the major source of MMP-9 is neutrophils and to a lesser extent also monocytes/macrophages (Westerlund *et al.* 1996; Pirilä *et al.* 2001). MMP-9 seems to be the major gelatinase in chronic periodontitis gingival tissue, dental plaque, saliva and GCF, and the GCF levels are elevated relative to LJP and healthy control GCF (Ingman *et al.* 1994a, b, 1996; Sorsa *et al.* 1994, 1995; Golub *et al.* 1995). In GCF collected with a mouthrinse method active gelatinase (MMP-9) was detected in the majority of periodontitis patients' samples (97.8%) but in only 11.4% of gingivitis samples (Teng *et al.* 1992). The mean gelatinase activity was highest in patients with recurrent loss of attachment and/or periodontal abscess formation and during periods of attachment loss, samples from the periodontitis group exhibited a two-fold increase of mean gelatinase activity. Seguier *et al.* (2001) suggested that MMP-9 could be a marker for the clinical severity of periodontal disease based on their cross-sectional study.

5.6.4. Other MMPs with a role in periodontitis

The **stromelysin** sub-family members **MMP-3** (stromelysin-1), **MMP- 10** (strolemysin-2), **MMP-11** (stromelysin-3) and macrophage metalloelastase (**MMP-12**) do not cleave collagen themselves but they act synergistically with collagenases and gelatinases, and they also degrade proteoglycans and BM components. Stromelysins can activate other proMMPs including MMP-1, -8, -9, and -13 (Kähäri and Saarialho-Kere 1999; Moilanen *et al.* 2003; Beklen *et al.* 2006). They are expressed by various cell types as fibroblasts, endothelial cells, keratinocytes, and chondrocytes. MMP-11 can be activated intracellularly like MMP-1 and -2 (Nagase *et al.* 2005). GCF stromelysin in cascade together with MMP-8 and -9 levels differentiate periodontitis sites from healthy sites and have been found to correlate moderately with clinical indices (Haerian *et al.* 1995; Beklen *et al.* 2006).

MMP-3 (stromelysin-1) is produced by gingival connective tissue cells and it plays a significant role in the activation cascade of latent proMMPs -1, -8, -9, and -13 (Kähäri and Saarialho-Kere 1999; Moilanen *et al.* 2003). MMP-3 can degrade a wide range of extracellular matrix components including fibronectin, laminin, elastin and gelatin. In a longitudinal study Alpagot *et al.* (2001) detected significantly higher levels of MMP-3 and its endogenous inhibitor TIMP-1 in periodontitis sites than in GCF from healthy sites, and the levels were higher in progressing than in non-progressing sites. Thus MMP-3 was found to correlate with clinical findings, and it was concluded that sites with high levels of MMP-3 and TIMP-1 together with MMP-8 and -9 are at risk of progression of periodontitis (Beklen *et al.* 2006). This is in accordance with the finding by Haerian *et al.* (1995) who found that the mean amounts of stromelysins and TIMP-1 in diseased sites (gingivitis and periodontitis) were significantly higher than the mean amount of these GCF components in healthy sites, and GCF stromelysins and TIMP-1 differentiated healthy from diseased sites both correlating moderately with clinical indices. However, Ingman *et al.* (1996) found only relatively low levels of MMP-3 in AP as well as in LJP GCF and low levels of TIMP-1 in AP GCF.

The matrilysin subgroup member **MMP-7** (**matrilysin-1**) is synthesised by epithelial cells and it can degrade several ECM and BM components (for example collagens type IV and IX, laminins and fibronectin) as well as activate TNF-α, and several proMMPs including proMMP-8 (Balbin *et al.* 1998). It may be related to epithelial migration (Dunsmore *et al.* 1998) and in the normal physiology and antibacterial defence of junctional epithelium (Uitto *et al.* 2003). Matrilysin is expressed in the suprabasal cells of the normal human junctional epithelium facing the teeth and in epithelial cell rests of Malassez (Uitto *et al.* 2002). MMP-7 has been detected in periodontitis-affected GCF and peri-implant sulcus fluid (PISF) correlating with MMP-8 reflecting especially the severity of peri-implantitis (Tervahartiala *et al.* 2000; Kivelä-Rajamäki *et al.* 2003a; Sorsa *et al.* 2004a, b).

Membrane type matrix metalloproteinases (**MT-MMPs**) are not, like other MMPs, usually secreted as soluble proforms, but instead they mainly exert their activity on the cell surface. However, cultured gingival fibroblasts from periodontitis patients can express and release a soluble form of MMP-14 (Cox *et al.* 2006), which can be detected in periodontitis affected GCF (Tervahartiala *et al.* 2000) and inflammatory tear fluid and bronchoalveolar lavage fluid (Maisi *et al.* 2002; Holopainen *et al.* 2003). **MMP-14** can activate other MMPs among them proMMP-8 and -13, and it has been detected in fibroblasts from inflamed

human gingival specimens (Dahan *et al.* 2001; Holopainen *et al.* 2003; Cox *et al.* 2006). MMP-14 can degrade, among other substrates, collagens I—III and thus activate proMMP-2, -13, and -8 (Sternlich and Werb 2001). Also enhanced levels of leukolysin (MT2-MMP or MMP-25) together with matrilysin-2 (MMP-26) have been detected in periodontitis-affected GCF (Emingil *et al.* 2006a).

5.7. GINGIVAL CREVICULAR FLUID (GCF)

GCF is an important local defence mechanism of the periodontal pocket. Production of GCF is directly related to the dilatation and increased permeability of blood vessels in the gingival connective tissue. In the healthy periodontal sulcus GCF is an exudate of serum origin and excreted in small amounts. With an increase of the severity of periodontal inflammation the amount of GCF excretion increases significantly (Armitage 1995). Because most clinical periodontal indices are somewhat subjective in nature, quantification of GCF volume has been used as an objective measurement of periodontal tissue inflammation in scientific research and to evaluate the inflammatory status of periodontal tissues (Brill 1960).

In inflamed gingiva GCF is an inflammatory exudate derived from a number of sources, including serum, the connective tissue and epithelium through which GCF passes on its way to the gingival crevice. As fluid traverses the inflamed tissue, enzymes and other molecules as well as products of cell and tissue remodelling and degradation of host and bacterial origin are collected. The cellular components of GCF are 70—80% neutrophils, 10—20% monocytes/macrophages, 5% mast cells and 5% T-lymphocytes.

The rich array of cellular and biochemical mediators in GCF reflect the metabolic status of periodontal tissues. GCF has the benefit of being closely approximated to the specific site of periodontium thus providing more information about periodontal status than markers for example in serum or urine. Though the risk for periodontal disease is patient based, the progression of periodontitis takes place on a tooth site level (Champagne *et al.* 2003). Thus GCF offers the possibility to monitor the pathologic processes of destructive periodontitis lesions site specifically and provides diagnostic information beyond that provided by the traditional clinical examination (Lamster *et al.* 1985).

Decreased GCF flow in smokers has been reported (Hedin *et al.* 1981; Persson *et al.* 1999). However, McLaughlin *et al.* (1993) found that following smoking there was more than a two-fold increase in GCF output compared to sham smoking (smoking movements with an unlit cigarette), and the heaviest smokers produced highest GCF volumes. In periodontally healthy subjects after smoking cessation GCF flow increased to comparable levels with non-smokers suggesting that the gingival microcirculation recovers in a few days after smoking cessation in periodontal health (Morozumi *et al.* 2004).

5.7.1. Potential disease markers in GCF

Great interest has focused on gingival crevicular fluid in the determination of current periodontal status and as a possible predictor of periodontal disease progression. GCF has been analysed since the 1930's to identify biomarkers of periodontal disease activity. Studies have shown that there is a generalised increase in GCF protein content at sites of active infection as well as an increase in GCF production. Over 65 components have been preliminary examined with the purpose to find a possible marker or markers which could identify the sites that are in the risk of periodontal disease progression, i.e. additional loss of attachment (Armitage 2004).

One problem to be solved when biomarkers in GCF are used for diagnostic purposes is the diagnostic potential of the biomarker measured as a concentration in GCF or as total amount in the sample (see also **Tables 1** and **4**). GCF volume increases in order of healthy, gingivitis, and periodontitis (Nakamura *et al*. 2000) and the volume of GCF recovered with paper filter strips is directly related to the periodontal pocket probing depth (Gamonal *et al.* 2000a). However, increased GCF flow does not necessarily reflect the periodontal disease activity. The concentration of biomarkers in GCF can be affected by the volume. Excess GCF flow, especially in an inactive site may dilute the concentration of the biomarker (Gamonal *et al.* 2000a). Though significantly higher GCF volumes and amounts of total proteins in GCF samples are obtained from periodontitis patients compared with control subjects (Vernal *et al.* 2005) neither total amount nor concentration of individual biomarker in GCF necessarily correlates with periodontal parameters (Gamonal *et al.* 2000a). In the case of some biomarkers a correlation may be found for either detection method (Nakamura *et al*. 2000; Vernal *et al.* 2005).

GCF can be harvested from the gingival pocket with paper filter strips or micropipette tubes using an intracrevicular washing method. The intracrevicular washing method for collection of gingival crevicular components (cells and chemical components) was introduced by Salonen and Paunio (1991), and the method has further been modified by Figueredo and Gustafsson (1998). In the method a small amount of washing solution is manually ejected through a tiny needle into the bottom of the gingival crevice, and the solution is immediately collected with continuous aspiration at the gingival margin with a collecting needle into a sample tube.

The collection time influences the composition of the collected GCF and a long sampling time especially with micropipettes, may irritate the tissue and enhance the vascular permeability and change the composition of GCF. Sampling time of 30 s or less is recommended (Eley and Cox 1998a) but variable sampling times from 5 s (Talonpoika and Hämäläinen 1994) to 3 min (McLaughlin *et al.* 1993) can be found from literature reviewed in this thesis. With paper filter strips, most of the enzymatic activity of GCF is depleted during the first few seconds of sampling, and more than 10 min is required before equilibrium is regained (Persson and Page 1990).

5.7.1.1. Host derived enzymes and their inhibitors in GCF

5.7.1.1.1. Proteinases

Most of the potential periodontal disease markers in GCF originate from neutrophils, monocytes/macrophages, mast cells and T lymphocytes; the first two mentioned being the most essential part of the host's inflammatory response in GCF. Proteinase enzymes are endopeptidases which are resident in inflammatory cells but also in tissue cells. All of them can degrade ECM and BM proteins *in vivo*. They are grouped into four groups based on the biochemical nature of the active site: 1) serine proteinases, 2) matrix metalloproteinases (already discussed in the previous section), 3) cysteine proteinases, and 4) aspartate proteinases. The first two mentioned groups are most active at neutral pH (neutral proteinases) and they mediate most extracellular *in vivo* proteolytic events. Cysteine proteinases and aspartate proteinases are optimally active at acidic pH in phagolysosomes (intracellular proteolysis), but these acidic proteinases can take part in extracellular

proteolysis if they are released at sufficiently high concentration into an acidic environment (Owen and Campbell 1999a).

Neutrophils form the first line of defence against a bacterial challenge in the periodontal pocket, and they have a highly protective role in inflammatory reactions. Over 90% of leukocytes in GCF are neutrophils (Uitto *et al.* 2003; Sorsa *et al.* 2004a, b). Leukocyte derived proteinases have the capacity to degrade almost all components and matrices of the ECM and basement membranes as well as extracellular proteins like complement components, clotting factors, immunoglobulins, and pro- and anti-inflammatory mediators (Owen and Campbell 1999a, b; Gueders *et al.* 2005). If the control of the activity of these enzymes is disturbed, they contribute to tissue injury in several organs as well as in the periodontium. Neutral proteinases from neutrophils also facilitate neutrophil migration and help to create an environment for effective defence against microbes (Uitto *et al.* 2003). Thus, neutrophil proteinases (MMP-8 and -9) can exert anti-inflammatory protective characteristics (Owen *et al.* 2004; Sorsa *et al.* 2004a; Gueders *et al.* 2005).

The proteinase enzymes found in the GCF which are associated with the pathogenesis of periodontitis and which may be of possible diagnostic value are shown in **Table 1**:

- **Neutrophil elastase**, a **serine proteinase** that is released from neutrophil azurophilic granules during phagocytosis stimulation and cell lysis. Elastase, like other serine proteinases, has broad substrate specificity, and it is capable of degrading a variety of human tissue molecules such as collagens, laminins, fibronectin, proteoglycan and elastin, and it may have some direct anti-microbial effects. The superior correlation of total elastase activity compared to concentration, with clinical parameters of periodontal disease has been shown in several studies.
- Human **serine proteinase cathepsin G** may contribute to periodontal tissue destruction directly or via proteolytic activation of latent neutrophil procollagenase, and it may regulate vascular permeability and monocyte chemotaxis (Owen and Campbell 1999b). Cathepsin G can activate proMMP-8 and -9 (Kähäri and Saarialho-Kere 1999). Thus part of the cathepsin G can exist as an uncomplexed, free and functionally active form and thus reflect the disease process of gingiva (Tervahartiala *et al.* 1996).

- **Plasminogen activators** are two types of **serine proteinases**, the tissue/blood vessel type (t-PA) and the urokinase type (u-PA). Both of these are expressed in fibroblasts (Dano *et al.* 1985; Saksela 1985) and u-PA in gelatinase (tertiary) granules and primary granules in neutrophils (Borregaard *et al.* 1995). This system acts in both physiological (cell migration, wound healing, tissue remodelling, fibrinolysis, and inflammation) and pathological (malignant cell invasion) processes and is important in extracellular proteolysis by converting the proenzyme plasminogen into the broad-spectrum serine proteinase plasmin which in turn can activate certain latent proMMPs (Kruithof 1988).
- Lysosomal **cysteine proteinases cathepsin B, H and L** which have been localised to fibroblasts, monocytes, macrophages, and neutrophils.
- **Aspartate proteinase cathepsin D** which is released at the endpoint of inflammation.
- **Myeloperoxidase (MPO).** The myeloperoxidase-hydrogen peroxidase-chloride system with antimicrobial activity from neutrophil primary granules has been implicated as part of innate host defence.
- **Beta-N-acetyl-hexosaminidase (ß-NAH)**, a lysosomal acid hydrolase released into GCF subsequent to phagocytosis by the neutrophil.
- Neutrophil primary granule acid glycohydrolase enzyme **ß-glucuronidase (ß-G)** which is involved in the destruction of non-collagenous component of extracellular matrix
- **Lactoferrin** that is released from neutrophil secondary granules and acts directly on the bacteria to prevent their growth.
- **Alkaline phosphatase (ALP)** secreted by neutrophils and other inflammatory cells has a role in areas of high bone turnover.

Monitoring the combination of several biochemical parameters might provide more information in order to predict future periodontal disease progression compared to measurement of a single biomarker. However, in a longitudinal study of chronic periodontitis patients in maintenance care unspecified neutral protease activity detected in GCF appeared to differentiate bleeding sites exhibiting only chronic inflammation but not the attachment loss (Bader and Boyd 1999). Nakashima *et al.* (1996) monitored adult periodontitis patients for one year and measured a combination of protease enzymes (ALP, collagenase and β-G).

Table 1. Proteinase enzymes in GCF. For each study the main findings are indicated in the table. *Longitudinal study; ▲ activity; ● total amount; ■ concentration; ? status unclear.

	Identifies active sites	Correlation with clinical parameters or disease severity	No correlation with clinical parameters or disease severity	Elevated levels in periodon-titis	Correlation with inflam-mation	Agreement with microbio-logical data
Elastase						
Eley and Cox 1992a				▲		
Armitage *et al.* 1994*	▲					
Ingman *et al.* 1994b				▲		
Murray *et al.* 1995			■●			
Eley and Cox 1996a	▲■					
Meyer *et al.* 1997				▲		
Jin *et al.* 1999						▲
Yamalik *et al.* 2000		▲				
Herrmann *et al.* 2001					▲	
Jin *et al.* 2002				▲		▲
Cathepsin G						
Suomalainen 1992				▲		
Kunimatsu *et al.* 1995			▲	■		
Plasminogen activators						
Yin *et al.* 2000				■		
Cathepsin B, H and L						
Kunimatsu *et al.* 1990 (B, H, L)			▲	▲		
Eley and Cox 1992a, b, c (B)		▲				
Eley and Cox 1992a (B, L)		■▲				
Eley and Cox 1996b* (B)	■▲					
Chen *et al.* 1998 (B)		▲				
Cathepsin D						
Buchmann *et al.* 2002a*, b*				▲		
MPO						
Buchmann *et al.* 2002a*, b*				▲		
ß-NAH						
Buchmann *et al.* 2002a*, b*				▲		
ß-G						
Lamster *et al.* 1988		▲				
Grbic *et al.* 1995			▲			
Lamster *et al.*1994a		▲				
Lamster *et al* 1995*	?▲					
Grbic *et al.* 1999*					■	
Buchmann *et al.* 2002a*				▲		
Layik *et al.* 2000			▲	▲		
Lactoferrin						
Adonogianaki *et al.* 1993		(■)			■	
Gustafsson *et al.* 1994			■			
Murray *et al.* 1995		●	■			
Figueredo and Gustafsson 2000			■●			
ALP						
Chapple *et al.* 1994					■	
Nakashima *et al.* 1994				■●		
Chapple *et al.* 1999 *	●					

They found significantly elevated levels of studied proteases from active relative to inactive sites prior to attachment loss without any significant differences in GCF volume and clinical indices. When Hanioka *et al.* (2005) studied 12 potential biomarker substances in GCF by regression analysis to find a combination which identifies sites with periodontal destruction, the combination of IgA and neutrophil elastase provided the most satisfactory predictive model for diagnosing the periodontal disease status.

5.7.1.1.2. Proteinase inhibitors

Proteinase levels in gingival tissue are determined partly by the balance between the enzyme and its natural inhibitors. The level of enzyme in the tissue and in GCF depends therefore to some extent upon the enzyme-inhibitor balance.

A number of proteinase inhibitors as possible markers of periodontitis have been studied (**Table 2**):

- **α1-proteinase inhibitor**, most abundant of specific inhibitors, serpins that regulate the serine proteinase activity in tissues. It is produced mainly by hepatocytes but also by activated neutrophils.
- **α2-MG**
- **α1-antitrypsin (α1-AT**)
- **Tissue inhibitors of metalloproteinases** (**TIMPs**) which perform the extracellular control of interstitial collagenases and other MMPs. TIMP-1 especially is expressed by many cell types including host fibroblasts, keratinocytes, monocytes/macrophages, endothelial cells and osteoblasts (Meikle *et al.* 1994), and can also be detected in GCF.

Table 2. Proteinase inhibitors in GCF. For each study the main findings are indicated in the table. *Longitudinal study; (r) reverse correlation.

	Identifies active sites	Correlation with clinical parameters or disease severity	No correlation with clinical parameters or disease severity	Elevated levels in periodon-titis	Correlation with inflammation
α1-proteinase inhibitor					
Ingman *et al.* 1994b				■	
Meyer *et al.* 1997			■		
Buchmann *et al.* 2002b		■			
α2-MG					
Adonogianaki *et al.* 1996			■		■
Chen *et al.* 1998		■			
α1-AT					
Adonogianaki *et al.* 1996			■		■
TIMPs					
Haerian *et al.* 1995		(■)			
Ingman *et al.* 1996		■			
Alpagot *et al.* 2001*	■			■	
Tüter *et al.* 2002				■ (r)	
Pozo *et al.* 2005		■ (r)			

5.7.1.1.3. Other enzymes originating from host cells

- The levels of **aspartate aminotransferase (AST)**, cytoplasmic enzyme released from damaged or necrotic cells during inflammation, have been used by measuring it in serum as an indicator of cell death that occurs in a variety of systemic disorders such as those following myocardial infarction. It could also provide evidence of cell death within the periodontal tissues and of possible disease activity.
- **Calprotectin** is a cytosol protein expressed by neutrophils, monocytes, activated macrophages and squamous mucosal epithelia. It is known to have a broad spectrum of *in vitro* antimicrobial effects (Nisapakultorn *et al.* 2001a) and its expression inhibits bacterial binding to mucosal epithelial cells (Nisapakultorn *et al.* 2001b).
- **Creatine kinase** and **lactate dehydrogenase**
- **Glycosidase**
- **Dipeptidyl peptidase II- and IV** -like activity. (**Table 3**)

Table 3. Other enzymes in GCF originating from host cells. For each study the main findings are indicated in the table. *Longitudinal study, ▲ activity; ■ concentration.

	Identifies active sites	Correlation with clinical parameters or disease severity	Elevated levels in periodon-titis	Correlation with inflammation	Agreement with microbiolo-gical data
AST					
Persson *et al.* 1990*	▲				
Chambers *et al.* 1991*	■				
Atici *et al.* 1998			▲		
Smith *et al.* 1998			▲		
Kuru *et al.* 1999					▲
Wong *et al.* 1999					▲
Shimada *et al.* 2000		▲			
Kamma *et al.* 2001					▲
Creatine kinase and lactate dehydrogenase					
Atici *et al.* 1998			▲		
Glycosidase					
Beighton *et al.* 1992		▲			
Dipeptidyl peptidase II- and IV -like activity					
Cox *et al.* 1992			▲		
Eley and Cox 1992a			▲		
Eley and Cox 1995*	▲■				
Calprotectin					
Kido *et al.* 1999		■			
Lundy *et al.* 2000			■		
Nakamura *et al.* 2000				■	

5.7.1.2. Inflammatory and immune markers in GCF

Cytokines

Cytokines, described as local hormones or cell to cell messengers, are small soluble proteins or peptides. More than 100 cytokines are known, among them chemotactic cytokines (chemokines), interleukins, interferons, and tumour necrosis factors. Cytokines control cell growth and differentiation, stimulate (chemokines and pro-inflammatory cytokines) or subdue (anti-inflammatory cytokines, interferons) the inflammation and regulate the development of the antimicrobial immunity in cooperation with antigen presenting cells (Julkunen *et al.* 2003).

A summary of the following cytokines with a specific role in periodontitis is presented in **Table 4**:

- **IL-1,** a cytokine with pro-inflammatory effects and a potent stimulator of bone resorption is produced by activated macrophages, monocytes, B cells, neutrophils, fibroblasts, and epithelial cells. It is involved in pro-inflammatory process, matrix degradation, and wound healing (McCauley and Nohutcu 2002).
- **IL-8** is a potent chemotactic and activating factor for neutrophils, and it is thought to provide protection against periodontal infections by enhancing host defence mechanisms.
- **IL-6** is a macrophage and T-cell originating cytokine. Healing of periodontal pockets may be affected by an accumulation of IL-6 in the adjacent connective tissue as a result of increased synthesis or reduced release into the GCF (Guillot *et al.* 1995).
- **TNF-α** is a monocyte/macrophage-derived pro-inflammatory cytokine which can stimulate proteolytic enzyme synthesis and osteoclast activity.
- The chemokine **RANTES**, a chemoattractant cytokine of T-cell origin is a potent chemoattractant for inflammatory cells and may play a role in host response in active inflammation.
- **Receptor antagonist of IL-1 (IL-1ra)**

Immunoglobulins

Potential immune mediators relevant to periodontal pathology are **immunoglobulins**. Antigens from periodontopathogenic bacteria induce antibody production, and both serum and locally produced antibodies can be detected in GCF. However, with the current knowledge neither specific antibody nor total GCF-Ig seems to be valuable in distinguishing between stable and progressing sites or patients at risk (**Table 5**). The role of IgA in GCF may be protective (Lamster *et al*. 1994b), and maintenance of high levels of IgA in GCF may protect from progression of periodontal tissue destruction (Grbic *et al.* 1999).

Ebersole and Cappelli (1994) identified antibody isotypes and subclass proportions of IgG in GCF in periodontitis patients with *A. actinomycetemcomitans* cultivated from subgingival plaque. They found that over 95% of sites with elevated IgG4 were colonized with *A.*

actinomycetemcomitans, and less than 50% of sites with elevated IgG2 were positive for *A. actinomycetemcomitans*. Sites infected with *P. gingivalis* in patients diagnosed to suffer of

Table 4. Cytokines in GCF. For each study the main findings are indicated in the table.
* Longitudinal study, ● total amount, ■ concentration; (r) reverse correlation; refr. refractory periodontitis.

	Identifies active sites	**Correlation with clinical parameters or disease severity**	**No correlation with clinical parameters or disease severity**	**Elevated levels in periodontitis**
IL-1 (α, β)				
Masada *et al.* 1990				■
Reinhardt *et al.* 1993			■	
Holmlund *et al.* 2004				■
IL-1α				
Mathur *et al.* 1996				■●
IL-1β				
Wilton *et al.* 1993			■	
Hou *et al.* 1995		●	■	
Lee *et al.* 1995b*	■			
Tsai *et al.* 1995		●		
Salvi *et al.* 1997		■		
Figueredo *et al.* 1999			■	■
Gamonal *et al.* 2000b*	■	●		
Rawlinson *et al.* 2000		■		
Engebretson *et al.* 2002*		■		■
Rawlinson *et al*. 2003		■ (r)		
Goutoudi *et al.* 2004*		●		
IL-1ra				
Rawlinson *et al.* 2000, 2003		■ (r)		
Holmlund *et al.* 2004				■
IL-8				
Tsai *et al.* 1995		●		
Mathur *et al.* 1996				● ■(r)
Ozmeric *et al.* 1998 (LJP)			■●	
Gamonal *et al.* 2000b*		(●)		■
Gamonal *et al.* 2001				■
Jin *et al.* 2002				■
IL-6				
Reinhardt *et al.* 1993 (refr.)		●		
Lee *et al.* 1995b* (refr.)	■			
Guillot *et al.* 1995		■ (r)		
TNF-α				
Rossomando *et al.* 1990			■	■
Lee et al. 1995b*			■	
Tervahartiala *et al.* 2001 (tissue specimens)				■
RANTES				
Gamonal *et al.* 2000a	■●	■		●
Gamonal *et al.* 2000b*		(●)		■
Gamonal *et al.* 2001				■
Emingil *et al.* 2004a		■		

rapidly progressing periodontitis manifested high levels of specific antibody, levels correlated with clinical status and antibody levels decreased significantly after successful SRP with reduction of PD and resolving of inflammation (Johnson *et al.* 1993).

Other inflammatory mediators

Monocytic inflammatory mediator **prostaglandin E_2 (PGE_2)** is a metabolite of arachidonic acid and a potent biochemical mediator of inflammation with many proinflammatory effects. It can induce indirectly the destruction of ECM components of connective tissue by initiating vasodilatation, increasing capillary permeability and enhancing inflammatory cell infiltration. (**Table 5**)

Table 5. Immunoglobulins and other inflammatory mediators in GCF. For each study the main findings are indicated in the table. *Longitudinal study; (r) reverse correlation.

	Identifies active sites	**Correlation with clinical parameters or disease severity**	**No correlation with clinical parameters or disease severity**	**Elevated levels in periodontitis**	**Correlation with inflammation**
Immunoglobulins					
Total Ig					
Lamster 1992			■		
Page 1992			■		
IgA					
Grbic *et al.* 1995			■ (r)		
IgG subclasses (IgG1, IgG2, IgG3, IgG4)					
Sengupta *et al.* 1990			■	■	
Johnson *et al.* 1993		■		■	
Wilton *et al.* 1993			■		
Ebersole and Cappelli 1994				■	
Other inflammatory mediators					
PgE2					
Offenbacher *et al.* 1984*	■				
Offenbacher *et al.* 1986*	■				
Heasman *et al.* 1993					■
Nakashima *et al.* 1994		■		■	
Salvi *et al.* 1997		■			
Jin *et al.* 1999			■		
Leibur *et al.* 1999				■	
Preshaw and Heasman 2002			■	■	
C-reactive protein (CRP)					
Sibraa *et al.* 1991			■		

5.7.1.3. Tissue-breakdown products in GCF

Components of collagen breakdown products and other gingival connective tissue structures in GCF may serve as measures of periodontal connective tissue and basement membrane catabolism and have been considered as possible markers of bone resorption and periodontal disease activity.

Bone resorption markers

- **Osteonectin** (also known SPARC), a non-collagenous calcium-binding protein in the extracellular matrix in many tissues, is liberated from many different cell types as a heat shock protein, but may be related to inflammation in general (McCauley and Nohutchu 2002).
- **Osteocalcin (OC)** is a non-collagenous protein, which is a major component of bone extracellular matrix and mainly produced by osteoblasts. It can also be considered as a marker of bone turnover. Some of this protein is derived from circulation but osteocalcin may be produced also locally and released into GCF by osteoblast activity.
- **The pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP)** is a sensitive marker of bone resorption in osteolytic disease such as osteoporosis and osteoarthritis and a sensitive indicator of periodontal tissue destruction (Giannobile 1999).
- **Osteopontin (OPN)** is a glycosylated phosphoprotein in the bone matrix produced by several cell types including osteoblasts, osteoclasts and macrophages in sites of active bone metabolism. (**Table 6**)

Connective tissue and basement membrane degradation markers

The detection of components of degraded connective tissue or sulcular basement membrane which have passed to GCF could be indicative of tissue breakdown or active pocket formation associated with periodontal disease progression.

- **Laminin** is a glycoprotein found in all basement membranes**.** Emingil *et al.* (2004b, c; 2006b) showed that an adjunctive host response modulating low-dose doxycycline (LDD) therapy in combination with SRP therapy reduced GCF laminin-5 gamma2-

chain fragment levels together with MMP-8 levels and improved clinical periodontal parameters in patients with chronic periodontitis. Also in PISF laminin-5 gamma2-chain fragments have been found to correlate with clinical markers of peri-implantitis severity and PISF MMP-8 and -7 (Kivelä-Rajamäki *et al.* 2003a, b). The proteolytic processing of laminin-5 gamma2 chain in human inflamed periodontium is eventually conducted by human MMPs (Pirilä *et al.* 2001, 2003) since the fragmentation of laminin-5 gamma2 chain in periodontitis affected GCF (Emingil *et al.* 2004b, c, 2006b) resembles that done by human MMPs (Pirilä *et al*. 2003) and not that done by *P. gingivalis* trypsin-like protease (Emingil *et al.* 2006b).

- **Fibronectin** is concerned with the cell adhesion of connective tissue and can be found also in GCF.
- **Hydroxyproline**-containing peptides are released during fibrillar collagen degradation.
- The extracellular ground substance of connective tissue is comprised of high molecular weight aggregates, proteoglycans, constituted of **glycosaminoglycans (GAGs).** Connective tissue breakdown in periodontitis includes proteoglycan degradation with release of GAGs which pass into GCF. A rather large sample of GCF is needed (15 min sampling with micropipettes) to detect GAGs which reduces the value in chair-side diagnostics (Eley and Cox 1998b). Chondroitin-4-sulphate can also be detected from GCF of teeth undergoing orthodontic movement, showing evidence of trauma from occlusion, and from healing tooth-extraction wounds thus correlating with clinical conditions where degradative changes are occurring in deeper parts of periodontal tissues (Last *et al.* 1985).
- **Decorin** and **biglycan,** small proteoglycan metabolites. (**Table 6**)

Table 6. Tissue break-down products in GCF. For each study the main findings are indicated in the table. *Longitudinal study; (r) reverse correlation.

	Correlation with clinical parameters or disease severity	No correlation with clinical parameters or disease severity	Elevated levels in periodontitis
Bone resorption markers			
Osteonectin (SPARC)			
Bowers *et al.* 1989	■		
OC			
Kunimatsu *et al.* 1993	■		
Nakashima *et al.* 1994	■		
Lee *et al.* 1999		■	
ICTP			
Talonpoika and Hämäläinen 1994	■		
Oringer *et al.* 1998 (peri-implantitis)	■		
Palys *et al.* 1998	(■)		■
Al-Shammari *et al.* 2001*	■		
OPN			
Kido *et al.* 2001	■		
Connective tissue and basement membrane degradation markers			
Laminin			
Figueredo and Gustafsson 2000			■
Emingil *et al.* 2004b, 2006b			■
Kivelä-Rajamäki *et al.* 2003a, b (peri-implantitis)	■		
Fibronectin			
Lopatin *et al.* 1989			■ (r)
Talonpoika *et al.* 1993	■		
Huynh *et al.* 2002			■
Hydroxyproline			
Akalin *et al.* 1993		■	
GAGs			
chondroitin-sulphate			
Okazaki *et al.* 1995			■
Smith *et al.* 1995	■		■
hyaluronic acid			
Last *et al.* 1985		■	
Decorin and **biglycan**			
Waddington *et al.* 1998			■

5.7.1.4. Enzymes of bacterial origin in GCF

The ability of bacteria to directly damage periodontal tissues is limited though a number of periodontopathogenic bacteria produce several proteolytic enzymes that are important virulence factors for these microbial species. Thus part of the proteolytic activity in GCF can be ascribed to bacterial proteases while neutrophils, present in large numbers at inflamed sites, are mainly responsible for the proteolytic activity (Sorsa *et al.* 2004a, b). Enzymes from

P. gingivalis and *A. actinomycetemcomitans* can participate in periodontal disease progression by producing immunoglobulin degrading enzymes in LJP detectable in GCF (Gregory *et al.* 1992). Measurement of these enzymes in GCF is complicated because of their very broad substrate specificity, their rather uncharacterised protein structures, a lack of specific enzymatic assays for their measurements, and their tight association with bacterial cell membranes (Uitto *et al.* 2003).

Trypsin- and tryptase-like activity of can be detected in higher levels from chronic periodontitis GCF compared to healthy subjects (Eley and Cox 1992a). In addition, the presence of bacterial **dipeptidyl peptidase** originating from *P. gingivalis* has been detected in GCF (Gazi *et al.* 1995). A 2-year longitudinal study indicated that both bacterial **gingivain/gingipain** and dipeptidyl peptidase may be predictors of periodontal attachment loss with higher positive predictive values for gingivain/gingipain (Eley and Cox 1996c).

5.7.2. Effect of smoking in GCF biomarkers

Smoking appears to affect the normal balance of several cytokines as well as of other GCF biomarkers (**Table 7**). Smoking decreases mean GCF **IL-1α** concentrations significantly but does not affect mean total protein concentration of samples. Neutrophil numbers are not significantly different between S and NS suggesting that the reduced IL-1α concentration in S is independent of any effect of smoking on neutrophil chemotaxis, and smoking may directly inhibit IL-1α production (Petropoulos *et al.* 2004). Thus GCF IL-1α may be derived from the inflamed tissues rather than being locally produced by neutrophils in the gingival pocket.

The influence of smoking on neutrophil **elastase** activity under healthy or slightly inflamed conditions is limited (Persson *et al.* 1999). Contradictory findings concerning elastase in GCF of smoking subjects with periodontal disease have been reported. Lower concentrations of functional elastase in S than NS GCF led to the hypothesis that smokers' PMNs may release elastase prior to reaching the periodontal tissues, for example during passing through the lungs, or possibly a greater proportion of the elastase is bound to its substrate and remains undetected thereby complicating the diagnostic value of GCF elastase in smokers (Alavi *et al.* 1995).

It has also been concluded that decreased local levels of enzyme inhibitors may result in increased tissue damage due to an increased activity of elastase and collagenase (Persson *et al.* 2001). Following surgical treatment, in the S group the levels of GCF **α1-AT**, **α2-MG** and **MMP-8** remained unaltered, and in the NS group the levels decreased indicating a more favourable treatment outcome, and elastase remained unaltered in both groups (Persson *et al.* 2003). Söder (1999) showed that smokers' periodontal sites with high levels of MMP-8 were significantly deeper compared with sites with low levels. Moreover, a positive correlation between PD and levels of MMP-8 both in S and NS patients with refractory periodontal disease existed. Also, significantly higher **PGE_2** levels were detected in sites with high levels of MMP-8 than in those with low levels (Söder 1999). In a regularly maintained periodontitis population, levels of PGE_2 in S than NS GCF were significantly lower (Söder *et al.* 2002). On a population basis smoking as a risk of periodontal disease does not appear to correlate with an elevation of GCF-PGE_2 (Heasman *et al.* 1998).

A soluble form of **intercellular molecule-1 (sICAM-1)** is known to be elevated in the circulation of S compared to NS subjects (Koundouros *et al.* 1996). Conversely, in the GCF of subjects with periodontitis sICAM-1 was significantly lower in S compared with NS (Fraser *et al.* 2001). The difference in GCF concentrations of sICAM-1 compared with serum (4-fold in serum) was significant in S but not in NS subjects. sICAM-1 molecules may possibly bind to sequestrated neutrophils in periodontal microvasculature and provoke the release of neutrophil elastase. This results in inappropriate endogenous protease release, contributing to periodontal destruction in the vicinity of the gingival microvasculature.

Table 7. Effect of smoking on GCF content of some biomarkers in periodontitis or in experimental gingivitis*.

Biomarker	Effect in GCF	Reference
Host enzymes and inhibitors		
MMP-8	↑	Söder 1999
elastase (activity)	↑ / ↓	Söder 1999 / Alavi *et al.* 1995
elastase—α1-AT-complex	↑	Söder 1999
α1-AT	↓	Persson *et al.* 2001
α2-MG	↓	Persson *et al.* 2001
plasminogen activating system	—	Buduneli *et al.* 2005
Inflammatory and immune markers		
PGE_2	↑ / ↓ (in regular maintenance care)	Söder 1999 / Söder *et al.* 2002
TNFα	↑	Boström *et al.* 1998a, b, 1999
IL-4*	↑	Giannopoulou *et al.* 2003a
IL-4	↑	Giannopoulou *et al.* 2003b
IL-6	—	Erdemir *et al.* 2004; Boström *et al.* 1999
	↑	Giannopoulou *et al.* 2003b
IL-8*	↓	Giannopoulou *et al.* 2003a
IL-8	↑	Giannopoulou *et al.* 2003b
IL-10	↑	Goutoudi *et al.* 2004
IL-1β	—	Boström *et al.* 2000; Giannopoulou *et al.* 2003b; Goutoudi *et al.* 2004
IL1-ra	↓ / —	Rawlinson *et al.* 2003 / Boström *et al.* 2000
IL-1 α	↓	Petropoulos *et al.* 2004
sICAM-1	↓	Fraser *et al.* 2001
IgA and IgG	—	Boström *et al.* 2000
Bone resorption markers		
ICTP	↑	Al-Shammari *et al.* 2001

5.8. SALIVA AND ORAL RINSES IN PERIODONTAL DIAGNOSTICS

Saliva may offer potential as a periodontal diagnostic aid at the patient level, and for this purpose it has been a subject of much research activity. Saliva can easily be collected as whole saliva or from specific salivary glands with or without gustatory stimulation. Whole saliva is a mixture of oral fluids: it contains secretions of the minor and major salivary glands as well as GCF, excreted bronchial secretions, serum and blood cells from oral wounds, micro-organisms and desquamated oral epithelial cells and food debris (Kaufman and Lamster 2000). Salivary-based caries susceptibility tests have been relatively well established (Mandel 1993). Whole saliva holds promise for periodontal diagnostics because it contains locally delivered and systematically-derived markers of periodontal disease derived from GCF of all teeth. Periodontal treatment reduces salivary enzyme levels, and salivary enzyme levels have been proposed as a means to monitor the effectiveness of periodontal treatment (Zambon *et al.* 1985).

Aminopeptidase enzyme activity in mixed whole saliva is significantly higher in LJP and AP patients compared to healthy controls (Nakamura and Slots 1983; Gregory *et al.* 1992). AP patients demonstrate higher levels of salivary **collagenase** and **elastase-like activity** when compared with LJP patients and healthy controls (Uitto *et al.* 1990; Ingman *et al.* 1993). Very little collagenase activity was found in whole saliva of edentulous subjects suggesting that most of the collagenase in saliva is derived from neutrophil cells entering the oral cavity through the gingival crevice (Sorsa *et al.* 1990; Uitto *et al.* 1990). Most of the periodontitis patients' salivary collagenase is in an active form and in periodontally healthy subjects in a latent form (Uitto *et al.* 1990; Hayakawa *et al.* 1994). Total **TIMP-1** in periodontitis patients' whole saliva seems to be lower than in healthy subjects (Hayakawa *et al.* 1994).

Periodontal therapy resulted in reduced **collagenase** and **elastase** activities in chronic periodontitis and LJP patients' saliva (Gangbar *et al.* 1990; Uitto *et al*. 1990; Ingman *et al*. 1993) as well as in adult periodontitis patients' oral fluid (Uitto *et al.* 1996). Salivary elastase was found to correlate significantly with the number of deep periodontal pockets and the percent of bleeding sites (Nieminen *et al.* 1993). There is also a finding that S periodontitis

patients had significantly lower salivary elastase levels than former S or NS periodontitis patients and less neutrophils in saliva than NS patients (Pauletto *et al.* 2000).

Also, salivary **proteolytic activity** and **MMP-8** levels were lower in current smokers than in former smokers but there was no difference in BOP. It was concluded that the use of salivary/mouthrinse proteinases as diagnostic markers of periodontal disease activity should be done with caution (Liede *et al.* 1999). High **MMP-9** (92 kDa gelatinase) levels were detected in periodontitis patients' whole saliva compared with healthy subjects (Mäkelä *et al.* 1994).

Also salivary **MPO** activity has been suggested to have a relationship to the severity of periodontal disease, while increased MPO activity has been detected in saliva of patients with rapidly progressing periodontitis (Over *et al.* 1993). Lower levels of **lysozyme** has been reported in periodontitis patients' saliva compared with healthy controls (Markkanen *et al.* 1986) and increased **peroxidase** activity in experimentally induced gingival inflammation (Smith *et al.* 1984). However, **lactoferrin** concentrations in chronic periodontitis patients' stimulated and unstimulated whole saliva and GCF were reduced significantly after periodontal surgery, whilst no change in salivary peroxidase or lysozyme could be detected (Jentsch *et al.* 2004).

From plasma cells in the salivary glands secretory **IgA** is the main specific immune mechanism of saliva. **IgG** is present in low concentrations in saliva and primarily derived from serum via GCF. Studies evaluating the presence of total and specific immunoglobulin directed against periodontal pathogens in saliva have aimed to determine their relationship to periodontal status. Higher levels of IgA have been detected in whole saliva of patients with gingivitis or periodontitis than in healthy subjects (Guven *et al.* 1982) but also a contradictory finding exists (Bokor 1997). Reductions in salivary IgA and IgG levels proved to be an indicator of periodontal treatment response (Reiff 1984), but in another study only IgG salivary concentration was elevated in untreated periodontal patients compared to post-treatment levels (Basu *et al.* 1976).

Specific immunoglobulins against periodontal pathogens hold more promise than assessment of non-specific immunoglobulin isotypes. Longitudinal studies support the potential of characterising specific salivary immunoglobulins as markers of periodontal disease activity

(Sahingur and Cohen 2004). Purified antigen preparations are suggested to give better sensitivity and specificity of antibody-based diagnostics because of reduced non-specific cross-reactivity with non-oral bacteria which share common epitopes with periodontopathogens (Offenbacher *et al.* 1993). Significantly higher concentrations of salivary IgG were found both in patients with AP and with JP towards *A. actinomycetemcomitans* but salivary IgA was not influenced by periodontal status (Sandholm *et al.* 1987). In addition to IgG, IgA specific against *A. actinomycetemcomitans* correlated significantly with corresponding antibody titers in the serum (Nieminen *et al.* 1993).

Saliva has been studied and proposed also as a potential media for detection of oral bacteria because of its non-invasive sampling (Kaufman and Lamster 2000). However, salivary analysis is mainly a research tool because of the lack of uniform data which supports the periodontal diagnostic value of salivary testing. Also it is not known how various general diseases such as HIV may affect values of salivary/oral rinse biomarkers for periodontal diseases (Mellanen *et al.* 1996, 1998). Furthermore, the effects of systemic medication of proteinase inhibitors such as doxycycline and bisphosphonates can be monitored successfully from saliva by zymography, collagenase activity assay, MMP-immunoassays and specific peptide activity assays for MMPs (Lauhio *et al.* 1994a, b, 1995; Nordström *et al.* 1998; Valleala *et al.* 2000; Fingleton *et al.* 2004).

5.9. DETECTION METHODS FOR GCF BIOMARKERS

5.9.1. Laboratory methods for GCF biomarker detection

The presence of proteolytic enzymes in GCF has been measured by **immunological assays** such as enzyme-linked immunosorbent assay (ELISA) (Ingman *et al.* 1996) and immunoblotting (Sorsa *et al*. 1994, 2004a, b; Kiili *et al.* 2002), both of these using high-affinity antibodies to recognise the given enzyme. Immunoblotting used with chemoluminescence is a capable and sensitive method for the estimation of molecular forms and mass of the enzyme of interest, but it is not very useful for quantitative analyses. ELISA/IFMA-methods can be used to test multiple markers and their levels in single samples. Two different antibodies are used: one for capturing the soluble enzyme and the other to detect the captured enzyme.

Substrate assays measure primarily the ability of the enzyme to degrade the substrate, and the enzyme activity will be estimated by different methods like gelatine enzymography (Gangbar *et al.* 1990), release of radioactivity from radiolabelled collagen fibrils (Villela *et al.* 1987), and fluorogenic assays based on synthetic peptides that mimic the cleavage site of collagenase (Knight *et al.* 1992; McCulloch 1994a, b). The degradation of the collagen in solution can be analysed by radiolabelled substrate assay SDS-PAGE fluorography (Larivee *et al.* 1986; Overall and Sodek 1987; Lee *et al.* 1991). This method needs seven days to be completed and it uses radioactivity and thus is unsuitable for clinical use.

The direct **measurement of** collagen or laminin-5 gamma2-chain **break-down products** in GCF and PISF has also been used as an analytical approach (Talonpoika and Hämäläinen 1994; Golub *et al.* 1997; Kivelä-Rajamäki *et al.* 2003; Emingil *et al.* 2004b, 2006b). A test based on biotinylation of collagen and the assay of labelled degradation products (soluble biotinylated collagen assay) can detect collagen and its labelled degradation products, and it was shown to be sensitive and reproducible in detection of protease activity in highly diluted mouthrinse samples (Mancini *et al.* 1999). Laminin-5 gamma2-chain fragments in GCF and PISF have been suggested to reflect active phase of periodontal and peri-implant inflammation (Kivelä-Rajamäki *et al.* 2003b; Emingil *et al.* 2004b, 2006b).

All of these methods are sensitive and specific and suitable to quantify collagenolytic enzymes/MMPs in microliter samples but all of them are time-consuming and not practical for routine clinical use.

5.9.2. Periodontal test kits

A number of test kits for the detection of GCF biomarkers by different methods or for the detection of certain periodontopathogens by an enzymatic reaction have been developed for commercial use or as a prototype test. In the following section the studies evaluating these tests are reviewed. However, with the available information (Internet) these tests, with the exception of BANA-test, are no longer available or not developed further from the prototype version.

A commercial test kit for measuring **AST activity** from GCF, PerioGard™ (Xytronyx, Inc., San Diego, CA), was designed to give a positive test score with GCF AST activity ≥ 800

µl/U. The test kit consisted of a tray with two test wells for the sample from each tooth and the reagents for conducting the test. The test result could be read by eye comparing the test colour to the positive control in 15 min. In a cross-sectional study the percentage of PerioGard™-positive sites decreased significantly between the baseline and post-treatment visits (Persson *et al.* 1995). With the same study design it was regarded to be an objective measure to distinguish between diseased and non-diseased sites and in monitoring the outcome of therapy (Magnusson *et al.* 1996). The predictive value of this test is unknown while the results of the longitudinal part of this multi-centre study have not been published.

Kamma *et al.* (2001b) found a high level of agreement between the periodontopathogens (*Streptococcus intermedius, Peptostreptococcus micros, B. forsythus, P. gingivalis, Campylobacter rectus, Selenomonas sputigena*) and positive AST test results (PerioGard™) at periodontal sites that were clinically considered potentially disease active. A kit based on same principle to measure GCF AST (Perimonitor™, Hawe Neos Dental, Switzerland) has been tested in a cross-sectional study, where positive test result correlated with gingival index (GI) but not with PD both in diabetic and non-diabetic patients suffering of chronic periodontitis (Yucekal-Tuncer *et al.* 2003). The same finding has been done in a study by Smith *et al.* (1998) where a positive AST test result (PerioGard™) correlated with GI but not with PD.

The third test based on the same principle: PocketWatch™ (Pacific Pharmaceuticals Inc., San Diego, CA), has been evaluated in a treatment study by Shimada *et al.* (2000). They concluded that AST levels monitored with this test may be a useful adjunct in the clinical assessment of periodontal disease sites though previously they found that the coefficients were too small to show a definite relationship between AST levels measured with PocketWatch™ and clinical periodontal status (Shimada *et al.* 1999). Barbosa *et al.* (2003) suggested that more research should be carried out to evaluate the true relationship between AST and periodontal disease because they found no correlation between GCF AST diagnosed with PocketWatch™ and the clinical parameters. In a 12-month longitudinal study the testing of GCF AST with PocketWatch™ chair-side assay in monitoring periodontal disease activity was associated with many false positive results, but a high negative predictive value was indicative of a periodontally stable site (Oringer *et al.* 2001). Elevated AST levels and test positive sites were present due to gingival inflammation with no disease

progression which diminishing the tests ability to discriminate between progressive and inflamed stable sites (Kuru *et al.* 1999).

A trypsin-like enzyme possessed by periodontopathogenic bacteria *B. forsythus*, *Treponema denticola* and *P. gingivalis* can be detected by an enzymatic assay where the substrate N-benzoyl-DL-arginine-2-naphthylamine (BANA) present on the test strips breaks down and produces a colour effect whose intensity is in proportion to the total amount of these microbial species (Loesche *et al.* 1990; Bretz *et al.* 1990). The BANA test requires a plaque sample from periodontal pockets. BANA hydrolysis has been shown to correlate well with PD in periodontitis sites (Grisi *et al.* 1998; Smith *et al.* 1998; Yucekal-Tuncer *et al.* 2003) but not with GI (Smith *et al.* 1998; Yucekal-Tuncer *et al.* 2003), and it's reliability to predict disease progression is weak (Hemmings *et al.* 1997). Loesche *et al.* have investigated BANA testing in monitoring the effect of systemic metronidazole in reducing spirochetes from deep periodontal pocket (1990) and in identifying the non-compliant patient with the usage of prescribed medication (1993), and found the test to be useful for these purposes. In a treatment study of chronic periodontitis patients with moderate to severe disease the BANA test Perioscan™ had a baseline sensitivity of 99% and specificity of 55% when related to clinical diagnosis. The probability of the test to agree with the clinical treatment outcome was calculated as 52%, leading to the conclusion that this diagnostic kit does not reliably reflect the clinical assessment of periodontal disease in cross sectional study or the treatment outcome (Hemmings *et al.* 1997). However, human/host-cell derived trypsin-like proteinases can easily degrade the BANA-substrate (Ingman 1994).

Hemmings *et al.* (1997) compared the Periocheck™ kit (a colorimetric assay for the presence of non-specific neutral proteases and elevated levels of proteolytic activity in GCF) with clinical methods. Baseline sensitivity for Periocheck™ was 88% and specificity 61% when related to clinical diagnosis, and the probability for agreement with the clinical treatment outcome was 50.4% thus not reliably reflecting the clinical assessment of periodontal disease. In an earlier study the highest percentage (33%) of elevated protease activity was found in patients clinically diagnosed as having advanced periodontal disease and the assay was reproducible with 98.6% in sites remaining stable with a 3-day testing interval (Bowers and Zahradnik 1989). After therapy, the neutral proteinase assay score decreased and the clinical parameters improved. However, the predictive value of neutral protease testing has not been evaluated in a longitudinal trial.

A prototype test kit for analysing GCF elastase has been evaluated in a longitudinal study (Palcanis *et al.* 1992; Armitage *et al.* 1994). After 15 s collection of GCF the test strip, impregnated with a human neutrophil elastase substrate is placed into an ultraviolet viewing box for visual assessment of the intensity of fluorescence, which is relative to elastase activity of the sample and scaled from 0 to 4 using intensity standards as a guide. In the earlier of these two studies visual interpretation of fluorescence in a strip was found to represent enzyme activity in GCF which presumably was related to biological changes occurring in the periodontal tissues site specifically (Palcanis *et al.* 1992). This VES (visual elastase scores) test gave a relatively low number of false negatives, but it failed as a discriminatory prognostic test because of its high percentage of false positives (Armitage *et al.* 1994). It was concluded that the VES test may, however, be of some advantage when used in conjunction with traditional assessments of periodontal disease.

5.9.3. Tests for genetic risk factors

Environmental exposures explain only in part the variability in susceptibility to periodontal diseases between individuals and part of the variation can be explained by genetic background (Michalowics 1994). Excess in pro-inflammatory cytokine production can lead to periodontal destruction at the site of bacterial plaque. Individuals with susceptibility to periodontal destruction have overactive monocytes which produce, when triggered by the presence of bacterial biofilm, pathologically excessive amounts of cytokines (Champagne *et al.* 2003). Individuals with a special IL-1ß genotype are found out to be more susceptible to develop periodontitis (Kornman *et al.* 1997b). Based on this discovery a blood test has been developed to determine the IL-1 genotype. Other candidate genes with a possible association to chronic periodontitis include for example TNFα polymorphism, human leucosyte antigens (HLA), and FcγR genotypes (Goteiner and Goldman 1984; Kobayashi *et al.* 1997; Galbraith *et al.* 1998). However, there can be several other factors which may contribute to individual susceptibility, and at the moment no specific genetic test can predict the development of periodontitis or the outcome of periodontal treatment.

Based on the studies of IL-1 gene cluster the Periodontal Susceptibility Test® (PST®), only commercially available genetic susceptibility test, has been developed. The PST® test evaluates the simultaneous occurrence of allele 2 at two different loci. When a patient is

diagnosed to have allele 2 in both of these loci, she/he is considered to be genotype-positive and produce more IL-1 in response to bacterial challenge. An IL-1 response predisposes to more inflammation and tissue destruction (Kornman *et al.* 1997b; Engebretson *et al.* 1999) and an individual is more susceptible to develop chronic periodontitis. The original finding was that among NS periodontitis patients, the composite phenotype was more frequent when compared to controls (Kornman *et al.* 1997b). The odds of being genotype positive were 6.8 times higher among severe periodontitis patients than among controls with mild or no disease. When smokers were included in the study the association became insignificant.

Later there have been studies that do not support association between IL-1 genotype and periodontitis (Mark *et al.* 2000) or inconsistencies with an association to periodontitis (Gore *et al.* 1998). Lack of consistency (Diehl *et al.* 1999; Parkhill *et al.* 2000) or no association (Hodge *et al.* 2001) has also been observed in studies of aggressive periodontitis leading to questions about the clinical applicability of the PST® test. Genotype-negative periodontitis patients have been reported (Kornman *et al.* 1997b; Cattabriga *et al.* 2001; Papapanou *et al.* 2001), and healthy periodontium or mild disease in genotype-positive patients has also been reported (Kornman *et al.* 1997b; Walker *et al.* 2000). Also, great racial variability exists in the prevalence of the composite IL-1 genotype (Kornman *et al.* 1997b; Gore *et al.* 1998; Armitage *et al.* 2000; Caffesse *et al.* 2002a). In predicting the outcomes of periodontal treatment (Ehmke *et al.* 1999; De Sanctis and Zucchelli 2000; Caffesse *et al.* 2002b) or implant failures (Wilson and Nunn 1999; Rogers *et al.* 2002) the PST® test was not accurate but there is debate about applicability of the test in predicting long-term stability during maintenance (McGuire and Nunn 1999; De Sanctis and Zucchelli 2000; Lang *et al.* 2000; Cattabriga *et al.* 2001).

5.9.4. Equipments for periodontal diagnosis

For the measurement of **subgingival temperature** ABIO-DENT (Danvers, MA) developed a Periotemp® thermal probe for the risk assessments of the early stages of periodontal disease. Haffajee *et al.* (1992a) measured baseline subgingival temperature and found that the deeper the pocket the higher the subgingival temperature was and correlated positively with plaque, gingival redness, BOP and CAL. In a longitudinal study it was found that an elevated subgingival temperature was related to subsequent attachment loss particularly in patients who exhibited several progressing sites (Haffajee *et al.* 1992b). Rams *et al.* (1993) postulated

that dental implant status can be monitored by sulcular temperature. The Periotemp® can also be used to evaluate the parameter from all sites of dentition. Nevertheless, the temperature probe has not been widely utilised in the periodontal clinic.

The Reflotron® System of Diagnosis is largely used in medicine in clinical laboratories to diagnose damage of the heart and liver by determining clinical-chemical parameters from capillary or venous blood, heparinised plasma and serum (Schuman *et al.* 2001). Cesco *et al.* (2003) studied using the Reflotron® the relationship between salivary AST levels and periodontal disease indicated by the community periodontal index of treatment needs (CPITN). Patients with CPITN 4 could be detected with this diagnostic system and periodontal destruction seems to be related to higher salivary AST levels.

Volatile sulphur compounds, such as hydrogen sulphide and methyl mercaptan, are toxic metabolites of periodontopathogens, and their role in periodontal disease is still poorly understood. The Diamond Probe / Perio 2000 System® (Diamond General Development Corp., Ann Arbor, MI) is a periodontal disease evaluation system to detect GCF sulphide concentration in various forms as an indicator of gram-negative bacterial activity. The system combines a Michigan dental probe with a sulphide sensor, and it measures PD, BOP and sulphide simultaneously (Zhou *et al.* 2004). Morita and Wang (2001) found in their study with chronic periodontitis patients that sulcular sulphide detected by using Diamond Probe / Perio 2000 correlated positively with the BANA test and may be an indicator of active periodontal disease. The same device was used by Zhou *et al.* (2004) in an experimental gingivitis study, and the results indicated that detection of sulphur was the first parameter to show plaque-induced gingivitis. Gleissner *et al.* (2002) regarded the sulphide monitor a reliable method and a valuable supplement to traditional clinical examination methods: no sulphide was detected in healthy controls while sulphide correlated significantly with PD in gingivitis, with CAL and PD in periodontitis, and with BOP.

6 AIMS OF THE STUDY

During periodontitis, tooth-supporting tissues (the gingiva, periodontal ligament, root cementum, and alveolar bone) undergo irreversible destruction which is primarily carried out by the inflammatory host response raised by the subgingival microflora. Periodontal destruction is considered to be episodic in nature with great individual variation in respect to the destruction pattern. This is rather difficult to diagnose with traditional clinical methods. Long-term treatment and prevention should be based on etiopathogenic factors, and for this purpose biochemical methods and markers may prove to be crucial.

The aims of this study were:

1. To assess the association of matrix metalloproteinase (MMP) -8 in GCF with periodontal disease (*I*) and to assess GCF MMP-8 levels during periodontal treatment and maintenance (*III*).
2. To develop a MMP-8 specific chair-side dip-stick test for identifying and monitoring the course and treatment of periodontitis (*II*).
3. To evaluate the value of GCF MMP-8 chair-side testing and the assessment of MMP-8 GCF levels with an immunofluorometric assay in monitoring treatment response and in identifying sites at risk of progression of periodontitis during the maintenance phase in chronic periodontitis patients (*IV, V*).
4. To evaluate the effect of smoking on GCF MMP-8 levels and chair-side test results (*V*).
5. To determine whether *Chlamydia pneumoniae* is localised in the periodontitis patients' deep periodontal pockets and its relation to GCF MMP-8 levels because *C. pneumoniae* is not normally associated with the periodontitis (*VI*).

7 MATERIALS AND METHODS

Detailed methods are described in the original publications *I—VI*.

7.1. Study populations and samples

Thirteen untreated chronic adult periodontitis patients participated in *study I*. GCF samples were collected for analysis of MMP-8 levels and elastase activities. Clinical parameters were recorded at baseline and two weeks after the completion of periodontal treatment (oral hygiene instructions and scaling and root planing, SRP).

In *study III* 20 untreated chronic adult periodontitis patients were recruited. GCF samples were collected from four sites in each subject with a pocket depth ≥ 5 mm for analysis of MMP-8 concentrations and clinical parameters. Recordings were done at baseline, after SRP and after a three month maintenance phase.

In *study IV* GCF samples were collected for MMP-8 concentration analysis and tested with a MMP-8 specific chair-side dip-stick test, and clinical parameters were recorded at baseline and after SRP from 11 untreated adult chronic periodontitis patients (90 sites), and once from 10 patients with gingivitis (58 sites) and from eight periodontally healthy controls (59 sites).

In *study V* 16 untreated chronic periodontitis patients participated in the cross-sectional treatment study [initial treatment response; baseline—post SRP; 11 smokers (S; 89 sites) and 5 non-smokers (NS; 43 sites)], and 15 of them [10 S patients (80 sites) and 5 NS patients (43 sites) were followed-up bimonthly during a 12 month maintenance phase (post SRP—12 months). At each visit GCF samples were collected from the same pre-selected sites for MMP-8 concentration analysis. Samples were tested with a MMP-8 specific chair-side dip-stick test and clinical parameters were recorded.

In *study VI* subgingival plaque samples were collected from 31 teeth of 12 adult chronic periodontitis patients. GCF samples for analysis of MMP-8 concentration were collected and clinical parameters were recorded. All samples and recordings were performed at baseline

and after SRP. Plaque samples from different pockets per patient were pooled to give one sample (Nieminen *et al.* 1995; Mellanen *et al.* 1996).

All periodontitis patients fulfilled the following criteria: 1) no history of systemic disorders, 2) no history of antibiotics and/or anti-inflammatory drugs within the past 6 months, 3) no history of any periodontitis treatment within the past 6 months, 4) the presence of at least 20 teeth, and 5) at least 5 sites exhibiting ≥ 4 mm probing depth and radiographic bone loss.

Gingivitis patients comprised cases with GI 2—4 and PD ≤ 3 mm. Periodontally healthy individuals had GI of 0 or 1 and PD ≤ 2 mm.

Approvals for the studies were provided by the Ethics Committee of the Institute of Dentistry, University of Helsinki, by the Ethics Committee of Glasgow Dental Hospital and School, and by King's Healthcare, London, United Kingdom. All patients gave informed consent for participation.

7.1.1. GCF and subgingival plaque sampling and clinical measurements

Prior to GCF sampling the crown of the tooth was gently cleaned to remove supragingival plaque, dried and isolated with cotton rolls to exclude contamination with saliva. The tip of a filter-paper sampling strip for absorbing GCF was placed into the gingival sulcus for 30 s avoiding any bleeding from the marginal gingiva, and removed into a test tube containing buffer solution (*I, III, IV—VI*). In *studies I* and *III* the volume of GCF was determined using a Periotron 6000.

For subgingival plaque sampling, supragingival plaque was removed from the teeth with a curette, gingival margins were dried with air and isolated from saliva with cotton rolls, and subgingival plaque samples were collected with sterile curettes. All samples of subgingival plaque were pooled to give one sample per patient (*VI*).

In studies *I, IV and VI* pocket depth (PD), bleeding on probing (BOP), gingival index (GI), and plaque index (PI) were recorded, in *study III* PD, BOP, PI, attachment loss (AL), suppuration (Supp), and modified gingival index (MGI), and in *study V* PD, AL and BOP. In

study III PD and AL measurements were done using the Florida probe with pocket depth and disc probes, respectively. In *studies I, IV, V* and *VI* clinical measurements were done with a manual probe.

The timeline and the site groups in *study V* are shown in **Figure 3.** Sites with an AL increase ≥ 2 mm during the maintenance phase (post SRP—12 months) were regarded as progressing sites and sites with repeatedly and exceptionally elevated MMP-8 concentrations (≥ 4000 µg/l two or more times during the maintenance phase) as high-responder sites (*V*). All but one of high-responder sites were smokers' sites. Therefore we examined smokers' sites grouped as high-responder and low-responder sites.

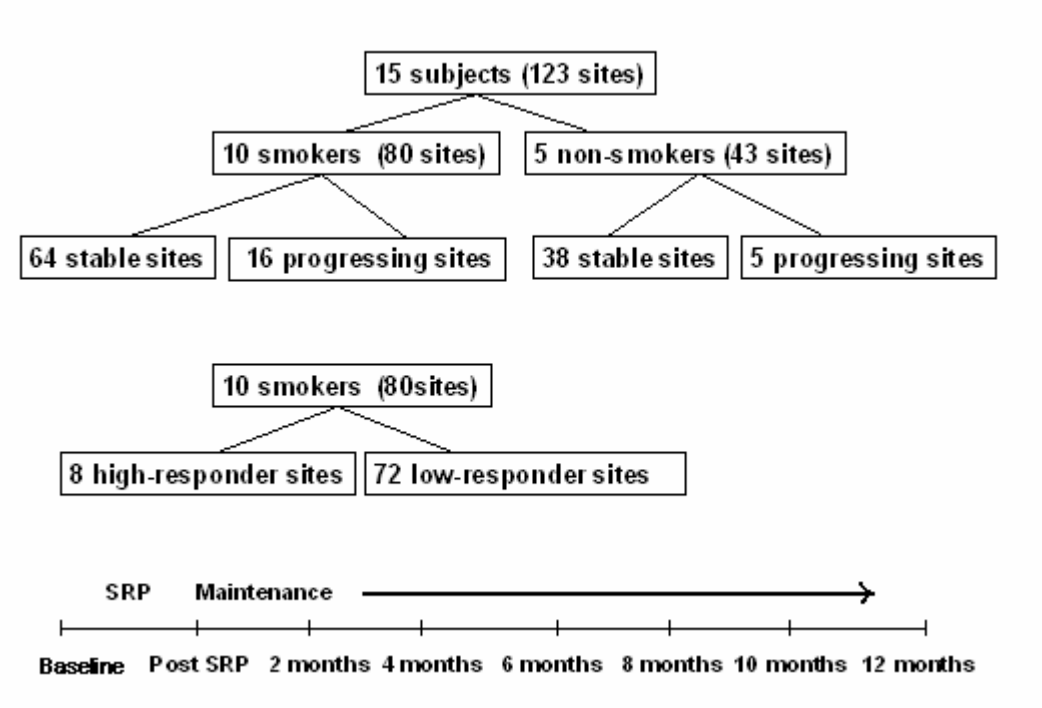

Figure 3. Groups and timeline of the longitudinal maintenance phase study.

7.2. MMP-8 SPECIFIC CHAIR-SIDE DIP-STICK TEST

Study II introduces the principle of the immunochromatocraphic MMP-8 specific chair-side dip-stick test.

7.2.1. Principle of the test

The MMP-8 test stick is based on the immunochromatography principle and it involves two monoclonal antibodies specific for different epitopes of MMP-8 (Hanemaaijer *et al.* 1997). Antibody 1 is bound to blue latex particles which act as the detecting label, and are dried next to the dip area of the dipstick. The other antibody is immobilised on a carrier membrane to catch labelled particles and to indicate a positive result (**Figure 4**). When the dip area of the test stick is placed in an extracted GCF sample, the dipstick absorbs liquid which flows up the nitrocellulose membrane. If the sample contains MMP-8 it binds to the antibody attached to the latex particles, and the particles are carried by the liquid flow along the test stick over the catching zone containing the other MMP-8 antibody. If the concentration of MMP-8 in the sample exceeds the cut-off value for the test (1000 µg/l), a blue line will appear to indicate a positive result. The test is designed to give a detection limit of 1000 µg/l based on earlier studies on MMP-8 in GCF collected from periodontitis and gingivitis patients' sites and from periodontally healthy individuals' sites. A second blue line confirms the correct performance of the test.

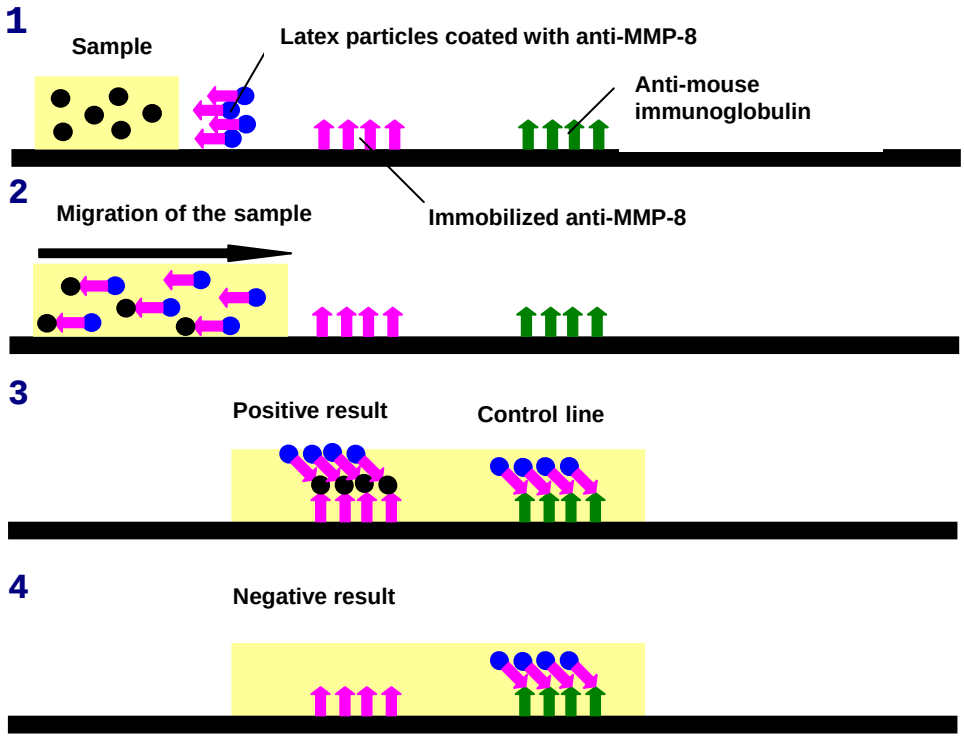

Figure 4. Principle of the MMP-8 specific chair-side test based on immunochromatography involving two monoclonal antibodies.

7.2.2. Chair-side procedure with the MMP-8 specific dipstick test

GCF sampling is described in chapter 7.1.1. After removal from the gingival sulcus, the sampling strip is placed for elution of the specimen proteins into a test tube containing 0.5 ml of HEPES-buffer, pH 7.4, for 5 min. The tip of an MMP-8 dipstick is then immersed in the buffer for about 10 s, removed and left for 5 min. The test is positive if the blue line appears (**Figure 5**). Positive test results were registered as: + (weak blue line), ++ (clear blue line), or +++ (strong blue line).

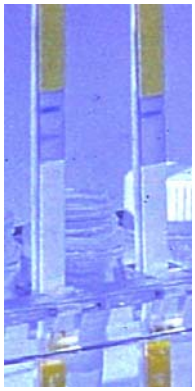

Figure 5. Test positive result of the MMP-8 specific chair-side dipstick test (left) is confirmed with the appearance of the blue line in the catching zone.

7.3. MMP-8 IMMUNOFLUOROMETRIC ASSAY (IFMA)

The concentration of the monoclonal MMP-8 of GCF was determined by a time-resolved immunofluorescense assay (IFMA) as described by Hanemaaijer *et al.* (1997). The monoclonal MMP-8-specific antibodies 8708 and 8706 were used as a catching antibody and a tracer antibody, respectively. The tracer antibody was labelled using europium-chelate (Hemmilä *et al.* 1984). The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 µM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/litre DTPA. Samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The monoclonal MMP-8 antibody specificity corresponded to that of the polyclonal MMP-8 antibody (Hanemaaijer *et al.* 1997).

7.4. ELASTASE ACTIVITY ASSAY

GCF eluates were assessed for elastase activity against the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-AFC by a fluorometric assay (Cox and Eley 1992). Eluates were added to 0.1 M assay buffer containing 0.1% v/v Triton X-100 and then the reactions started by the addition of substrate to give a final concentration of 10 μM in a volume of 2.0 ml. The concentration of liberated AFC was measured after 5 hours using a fluorostat calibrated with AFC. Total activities of strips were expressed in terms of μUnits (pmols of substrate hydrolysed per min). Enzyme activities were calculated relative to the corresponding volumes of GCF.

7.5. SUBGINGIVAL PLAQUE SAMPLE ANALYSIS FOR DETECTION OF *CHLAMYDIA PNEUMONIAE*

Subgingival plaque samples were examined for *Chlamydia pneumoniae* by means of PCR. DNA analyses were undertaken on the samples by means of the QIAamp DNA Mini Kit® (Qiagen, Germany), following the instructions of the manufacturer. A hot-start technique was used with *C. pneumoniae* OMP 1 gene probes. Europium-labelled probes were used in hybridization of PCR products. Counts of 50 000 or over were regarded as positive, counts of less than 50 000 as negative (Rintamäki *et al*. 2001).

7.6. STATISTICAL ANALYSES

Because of the skewed nature of the data, non-parametric tests were used. Comparisons of clinical parameters and concentrations of MMP-8 in GCF in various conditions were performed with the Mann-Whitney U-test (*IV, V*). The comparisons of clinical parameters and MMP-8 and elastase concentrations and elastase activities in the GCF samples before and after SRP were performed with the Wilcoxon matched pair's test (*I, III, IV, V*). Longitudinal comparisons of MMP-8 concentrations between different groups were analysed with one-way ANOVA after logarithmic transformation. In all comparisons the level of statistical significance was set at $p < 0.05$. Agreement between the quantitative determination of MMP-8 and the test stick result was estimated with the Kappa statistic (< 0.20 indicates poor and > 0.80 very good agreement) (*IV*) (Landis and Koch 1977).

8 RESULTS

8.1. MMP-8 CONCENTRATIONS IN GCF

MMP-8 levels in GCF dropped following initial periodontal treatment (*I, III, IV, V, VI*) and reduced still further by 50% compared with the post SRP situation once the wound healing phase of tissues was over (*III*).

In the cross-sectional study (*IV*) the median MMP-8 concentration in periodontally healthy subjects' GCF was 100 µg/l (interquartal percentile 40 µg/l, 170 µg/l), in gingivitis patients' GCF 470 µg/l (interquartale percentile 130 µg/l, 929 µg/l) and in adult periodontitis patients' GCF before treatment (SRP) 1850 µg/l (interquartale percentile 820 µg/l, 4290 µg/l). The difference between all conditions was statistically significant ($p < 0.001$). After treatment the MMP-8 concentration in the periodontitis patients' GCF decreased statistically significantly (median 970 µg/l, interquartale percentile 470 µg/l, 2220 mg/l; $p < 0.001$) but remained at a higher level than in periodontally healthy or gingivitis subjects' GCF.

The highest baseline GCF MMP-8 concentrations were in samples from periodontal pockets with PD > 5 mm and which were BOP positive (median 4300 µg/l, interquartale percentile 2600 µg/l, 6200 µg/l). Also in these sites SRP decreased MMP-8 concentrations significantly (median 1500 µg/l, interquartale percentile 480 µg/l, 3100 µg/l; $p < 0.001$) (*IV*).

8.1.2. Comparison of MMP-8 levels in smokers' and non-smokers' GCF (*V*)

The timepoints of GCF sampling and clinical measurements of the study are represented in **Figure 3**. In the longitudinal study (*V*) smokers' (S) sites (n = 80) and non-smokers' (NS) sites (n = 43) were analysed separately. In S sites the mean GCF MMP-8 concentration at baseline was significantly lower than in NS sites ($p < 0.001$). In S sites decrease of mean concentration after treatment (SRP) was not significant (baseline 1268 µg/l, after SRP 975 µg/l; $p = 0.7$) but significant in NS sites (baseline 3997 µg/l, post SRP 2076 µg/l; $p < 0.001$).

S and NS sites were further grouped according to the status at the end of the maintenance phase (*V*): S and NS sites with an attachment loss increase of ≥ 2 mm were regarded as

progressing sites and other sites as stable sites. 12% of NS sites (n = 5) and 20% of S sites (n = 16) were thus categorised as progressing sites. When progressing S sites and stable S sites were compared, the difference between mean MMP-8 concentrations was not significant at baseline, after SRP or at any maintenance visits, and in neither group of S sites SRP decreased mean MMP-8 concentration statistically significantly. However, in progressing S sites the distribution was, for every measurement, broader than in stable sites showing a tendency towards elevated MMP-8 levels. MMP-8 levels in progressing S sites were comparable with MMP-8 levels in progressing NS sites.

Sites with a MMP-8 concentration ≥ 4000 µg/l at two or more measurements during the 12 month maintenance phase were extracted from the data and analysed. Sites filling this criterion were eight S sites reaching exceptionally high MMP-8 levels (high-responder S sites; n = 8). These S sites concentrated into two patients, and five out of these eight sites were sites which also were categorised as progressing sites.

When high-responder S sites were compared with S sites with lower concentrations (low-responder S sites; n = 72), no significant difference in mean MMP-8 concentrations at baseline, post SRP and at 2-month measurement could be observed, but in high-responder sites the distribution was broader at these measurements. At 4—12-month maintenance phase measurements, difference between MMP-8 concentrations of these two groups of S sites were statistically significant.

8.2. MMP-8 CHAIR-SIDE TESTING

For a positive test-stick result the threshold for MMP-8 concentration was set at 1000 µg/l, and test results of individual sites were registered as follows: + (weak blue line), ++ (clear blue line) and +++ (strong blue line) (*IV, V*).

In the cross-sectional study *IV* the test stick result was well in unison with the quantitative MMP-8 analysis (IFMA) of the GCF samples (**Figure 6**). Only 18 of the 207 tested sites were in discrepancy. Thus the agreement was very good when calculated with the Kappa statistics ($\kappa = 0.81$), and the chair-side test provided a sensitivity of 0.83 and specificity of 0.96. The test-stick result also correlated well with the periodontal status of the above mentioned three groups. Only with few exceptions test results in healthy individuals' sites or

gingivitis patients' sites were negative. However, test result was most prone to be false negative or false positive near the concentrations of the cut-off threshold value 1000 µg/l (**Figure 6**) (*IV*).

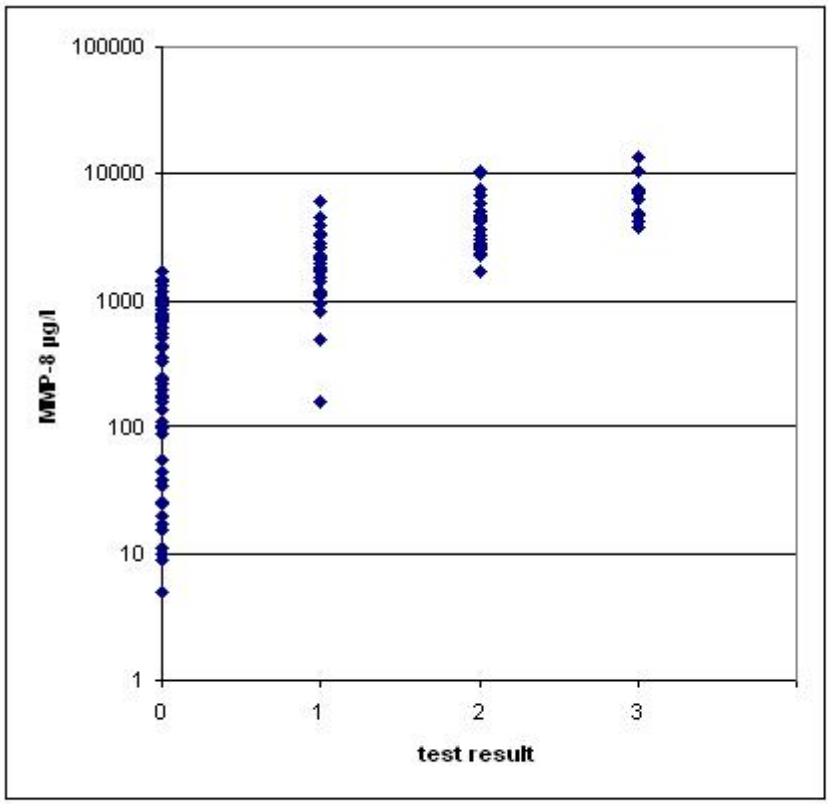

Figure 6. Scattergram of periodontitis patients' sites (n = 132) at baseline: correlation of MMP-8 concentration (IFMA µg/l) and MMP-8 specific chair-side dipstick test result (0 = negative, 1 = +, 2 = ++, 3 = +++) (Mäntylä *et al.*, to be published, 2006).

In the data processing of the longitudinal maintenance phase study (*V*) positive test results were expressed in two ways: all positive results + — +++ / clear test positive results ++ — +++. Percentages for respective test results were calculated. At the baseline measurement of the longitudinal study the MMP-8 chair-side dip-stick test result was positive in 50 % of sites when all positive test results were calculate and in 27% of sites when clear test positive results were calculated. After SRP the percentages were 39 and 10 respectively (n = 123) ($p < 0.05$).

Test result was more often positive at baseline in all NS sites (n = 43) and in progressing NS sites (n = 5) than in respective S sites (all S sites n = 80, progressing S sites n = 16). Test

positive percentages in NS sites decreased after treatment statistically significantly ($p < 0.001$). For all S sites the difference between baseline and post SRP test positive percentages was not statistically significant. In progressing S sites the percentage of test positive sites increased after SRP thus reaching proportion of NS sites which were test positive (*V*).

In high-responder S sites (n = 8) the baseline test positive percentages reacted poorly to SRP. In this group of sites higher test positive percentages were seen at the majority of measurement points during the maintenance phase than in low-responder S sites (n = 72) (*V*).

Based on test results of high- and low-responder S sites during the maintenance phase, sensitivity and specificity were calculated. Values were respectively 0.41 and 0.91.

8.3. BLEEDING ON PROBING SCORES

General BOP positive scores decreased after SRP when the smoking status was ignored (*I, III, IV, V*). Periodontitis patients' deepest pockets (> 5 mm) had highest BOP scores (89%) and the positive percentage of these sites decreased after SRP to 42% (*IV*).

When percentages of all positive BOP scores per group of sites during the maintenance phase (*V*) were calculated, percentages in S and NS sites with unstable character (progressing S and NS sites and high-responder S sites) were higher than in stable S and NS sites or in low-responder S sites. Positive BOP percentages were at a similar level in stable S and NS sites and low-responder S sites. BOP positive percentages in S sites with unstable character (progressing and high-responder S sites) were slightly higher than in progressing NS sites (*V*).

8.4. PD AND AL VALUES

PD values and GCF MMP-8 concentrations reduced significantly after SRP in studies *I, III, IV* and *VI* and in non-smokers' sites in study *V*. S sites' PD reduction as well as reduction of MMP-8 level after SRP was not statistically significant (*V*). S sites' and NS sites' initial PD was at the same level at the baseline (*V*). During the maintenance phase (*V*) PD levels of S sites remained higher than in NS sites.

In *study III* pocket reduction could still be observed at the three month maintenance visit and GCF MMP-8 levels were reduced by 50% compared with the post SRP situation.

AL decreased after SRP statistically significantly in sites with deep pockets (> 5 mm) (*IV*) and in the longitudinal maintenance study (*V)* when all sites (n = 123) were examined as one group. AL change was not statistically significant after treatment (SRP) in *study III*. During the 12-month follow-up period AL values did not differ statistically significantly between S and NS sites (*V*).

At all maintenance phase visits high-responder S sites' pockets were deeper than in the low-responder S sites. Baseline AL values were at similar levels in both high-responder and low-responder S sites, but the response of AL to SRP was statistically significant only in low-responder S sites.

8.5. ELASTASE IN GCF (*I*)

Total activities of elastase in GCF correlated significantly with GCF MMP-8 amounts. Also correlations with GI and BI before and after treatment, with PD before treatment and with PI after treatment were observed. However, when GCF elastase was measured as concentrations no correlations with clinical indices could be observed.

8.6. *CHLAMYDIA PNEUMONIAE* IN PERIODONTAL LESIONS (VI)

One patient was positive for *C. pneumoniae* in pooled subgingival plaque samples before periodontal treatment by SRP. The count in this patient's sample was negative after SRP. This patient did not differ from the other patients when quantitative results relating to other microbiological or enzymatic markers (MMP-8) of periodontal disease were observed.

9 DISCUSSION

To find a predictive biomarker with regard to progression of periodontal disease has been among the most desired ambition of biomarker researchers in periodontology. However, this purpose has revealed to be rather laborious and difficult. Optimally the biomarker should be able to differentiate ongoing connective tissue breakdown from inflammation of periodontal tissues. An ideal and predictive biomarker should be detected before the event of tissue breakdown, and it should remain at the site long enough so that it could be captured for analysis or for clinical testing. The most ideal situation would be that we should know the timescale from biomarker release to irreversible tissue damage. Besides, it should be possible to develop an easy to use chair-side test for clinical use based on its detection. For example, neutrophil elastase activity in periodontitis-affected GCF has been found to correlate with the clinical periodontal parameters but the correlation is not good when elastase is measured as concentrations (*I*) (Armitage *et al.* 1994; Murray *et al.* 1995). GCF collagenase activity test targets multiple collagenolytic MMPs (Kähäri and Saarialho-Kere 1999; Sorsa *et al.* 2004a; Ala-aho and Kähäri 2005) and for chair-side use it is inconvenient (Romanelli *et al.* 1999) when compared with an enzyme/MMP specific immunoassay which has been developed for the detection of MMP-8 (*I, II*).

Most biomarkers have been studied in a cross-sectional setting or in treatment studies. The results from follow-up studies are available for some biomarkers (for example **AST** Chambers *et al.* 1991, Persson *et al.* 1990, Oringer *et al.* 2001; **IgA** Grbic *et al.* 1999, **cytokines Il-1α, Il-1β, Il-1ra** Holmlund *et al.* 2004; **β-G** Lamster *et al.* 1995; **dipeptidyl peptidases II and IV** Eley and Cox 1995; **elastase** Palcanis *et al.* 1992, Armitage *et al.* 1994; **proteases** Bader and Boyd 1999; **collagenase** Lee *et al.* 1995a, Mancini *et al.* 1999; **lysosomal enzymes** Lamster *et al.* 1988; **combination of markers** Nakashima *et al.* 1996). In follow-up studies a major question of concern is which clinical periodontal surrogate parameter used in the clinic is a real or the best possible indicator of active periodontitis – a "golden standard" parameter for the progressing periodontitis which is not unambiguous. Manual periodontal probing has a relatively high measurement error. Thus, a 2—3 mm change in the clinical attachment level has been a demand for a confirmed progression of attachment loss (Page and DeRouen 1992; Armitage *et al.* 1994). However, the methods for assessing the progression of periodontitis do not agree well in identifying active sites with a

manual probe, automated probe nor by subtraction radiographic analysis, and a higher percentage of active sites can be detected with the manual probe than with the automated probe mostly because it is easier to manipulate the manual probe past the calculus retentions (Armitage *et al.* 1994). It has also been criticized that attachment loss as the parameter used to identify deteriorating sites is crude and relatively insensitive (Persson *et al.* 1995). The demand for 2—3 mm increase in attachment loss is an enormous amount of periodontal destruction and identifies only a small proportion of sites with progressing attachment loss. Therefore we used in our study also repeatedly elevated concentrations of GCF MMP-8 to identify sites of an unstable nature with possible minor attachment loss changes (*V*). Persson *et al.* (1995) proposed that a clinical trial comparing test outcome to active sites detected by attachment loss ≥ 2 mm would show a large proportion of apparently false positive results for sites which were categorised as stable because of the attachment loss less than 2 mm but which actually were active.

Evaluation of the prognostic value of a test is both practically and statistically very demanding. Complicated statistical methods and evaluations have not led to definitive conclusions about the predictive value of the studied biomarkers (Armitage *et al.* 1994; Eley and Cox 1995; Lamster *et al.* 1995; Persson *et al.* 1995; Bader and Boyd 1999; Oringer *et al.* 2001; Holmlund *et al.* 2004).

It is possible that different types of periodontitis release elevated levels of different biomarkers. Also, smoking is known to have a significant effect on GCF biomarker content. Regarding the enzymes in GCF, smoking affects the levels of MMP-8 and neutrophil elastase activity (Söder 1999). More than on enzymes, smoking seems to have effect on GCF inflammatory and immune markers (Boström *et al* 1998a, b, 2000; Söder 1999; Giannopoulou *et al*. 2003b; Goutoudi *et al*. 2004).

9.1. MMP-8 IN GINGIVAL CREVICULAR FLUID

MMP-8 is the major collagenase in chronic periodontitis patients' GCF (Sorsa *et al.* 1988, 1990, 2004a, b; McCulloch 1994a, b; Pozo *et al.* 2005) and its concentration, activity and degree of activation in GCF decreases significantly with meticulous hygiene phase periodontal treatment (Lee *et al.* 1995a; Kiili *et al.* 2002; Figueredo *et al.* 2004). This could be confirmed also in our study; the MMP-8 concentration correlated especially with the

depth of the periodontal pocket in bleeding sites (*IV*). This may in fact support the value of MMP-8 as an indicator of progressive periodontitis with advancing attachment loss instead of the inflammatory state. MMP-8, as all MMPs, has normal physiologic functions for example in wound healing and in connective tissue turn-over. In this regard, MMP-8 has recently been shown to exert protective anti-inflammatory effects in LPS-induced lung inflammation and allergen-induced granulocytic airway inflammation due to regulation of inflammatory cell apoptosis (Owen *et al*. 2004; Gueders *et al.* 2005). The decrease of GCF MMP-8 concentration during the three month maintenance phase even from the post SRP values (*III*) seemingly reflected participation of MMP-8 in periodontal tissue wound healing or defence reactions. The molecular forms/species of MMP-8 detected in periodontitis affected GCF correspond well with those detected in body's other inflammatory exudates such as bronchoalveolar lavage (Prikk *et al*. 2001, 2002), cerebrospinal fluid from young meningitis patients (Lindberg *et al.* 2006), tracheal aspirate (Cederqvist *et al.* 2003), sinus mucus samples (Kostamo *et al.* 2005), tear fluid (Holopainen *et al.* 2003; Määttä *et al.* 2006), aqueous humor from glaucoma patients (Määttä *et al.* 2005, 2006b), peri-implant sulcular fluid (Kivelä-Rajamäki *et al.* 2003a, b), and can represent as well, both pro-inflammatory or anti-inflammatory MMP-8 species (Owen *et al.* 2004; Sorsa *et al.* 2004a; Gueders *et al.* 2005).

The effect of smoking on GCF MMP-8 levels seems to be twofold. In our study MMP-8 levels tended to be lower in smokers' GCF when compared with non-smokers' GCF but only when all smokers' sites were examined as one group. The tendency to lower MMP-8 concentrations in smokers' GCF compared to non-smokers has also been observed by Persson *et al.* (2003) as well as lower salivary levels of MMP-8 in current smokers by Liede *et al.* (1999). However, in smokers' sites with progressing attachment loss during the maintenance phase MMP-8 concentrations were at the same level as in non-smokers' progressing sites. Moreover, when sites were explored in respect to repeatedly clearly elevated MMP-8 concentrations during the maintenance phase, in a fraction of smokers' sites MMP-8 concentrations reached the highest levels. In this group of smokers' sites GCF MMP-8 concentrations reached values over 10 000 µg/l while in non-smokers' sites the level of 5000 µg/l was seldom reached even in the group of sites which were considered as progressing (*V*). Thus, a lower level of MMP-8 in smokers' GCF does not apply to all smokers' sites or all smoking periodontitis patients.

Cigarette smoking aggravates various inflammatory tissue-destructive conditions (Bartecchi *et al*. 1994), among them periodontitis. Periodontitis with poor treatment response and outcome, i.e. refractory periodontitis, is more usual among smokers than non-smokers (MacFarlane *et al.* 1992; Magnusson *et al.* 1995; Magnusson and Walker 1996). Söder (1999) has detected a positive correlation between pocket probing depths and levels of MMP-8 in patients with refractory periodontal disease. Smokers with persistent periodontitis seem to have impaired granulocyte function with excessive release of protease enzymes (Söder *et al.* 2002). Smoking may interfere with the levels of protease inhibitors α2-MG and α1-AT which may be one mechanism by which smoking can affect the inflammatory process (Persson *et al.* 2001). Lowered neutrophil transcript levels in smokers were observed by Morozumi *et al.* (2004) which can be an indication of impaired neutrophils as a consequence of smoking. Petropuolos *et al.* (2004) studied IL-1α levels and the number of neutrophils in smokers' and non-smokers' GCF. No association between these two markers could be found between smokers and non-smokers either analysed by site or by patient. They concluded that smoking has no effect on neutrophil chemotaxis in the periodontium, and they further concluded that smoking may have specific actions on the inflammatory process but does not have a general inflammation suppressing effect. Gustafsson *et al.* (2000) observed that smokers' circulating neutrophils are more activated than non-smokers'. The increased membrane expression of receptor molecules in smokers' neutrophils indicates up-regulated functions, such as adhesion and degranulation. These findings support the hypothesis that the neutrophils from periodontitis patients who smoked may be the same in numbers than in non-smokers but hyper-reactive (Gustafsson *et al.* 2000). This is a possible explanation for excessive MMP-8 concentrations in some smokers' GCF (*V*). A two-fold increase in MMP-8 levels has recently been found from skin of smokers compared to non-smokers (Knuutinen *et al.* 2002). Our study indicated that more than two-fold higher levels of MMP-8 in smokers' GCF can be found when compared to non-smokers' GCF. This finding differs from the earlier observations about smokers' general lower GCF MMP-8 levels (Persson *et al.* 2003).

9.2. MMP-8 SPECIFIC CHAIR-SIDE TEST

Among those smokers who responded to periodontal treatment, GCF MMP-8 levels stayed at a lower level during the maintenance phase compared to non-smokers (*V*). However, a worse treatment response to SRP in smokers when compared with non-smokers was obvious in all

studied groups of sites. Smokers' sites with stable characteristics exhibited low prevalent GCF MMP-8 concentrations while progressing smokers' sites and high-responder smokers' sites exhibited even clearly elevated concentrations. Thus, an elevated MMP-8 level in a smoker's site is an indicator of instability and can be conveniently detected with the MMP-8 specific chair-side test (*V*). Among non-smokers the finding was not as clear, possibly as consequence of the low number of progressing sites, better treatment response, easy maintenance and to relatively low cut-off value (1000 µg/l) of the test (*V*).

The baseline sensitivity and specificity of the MMP-8 specific chair-side test related to clinical diagnosis was excellent: both sensitivity and specificity were very good when MMP-8 concentrations were compared with test positive results from periodontally healthy and gingivitis and periodontitis subjects (*IV*). However, sensitivity calculated during the maintenance phase would have been low for progressing smokers' and non-smokers' sites (*V*). This is a problem which arises from the nature of periodontitis as well as from the definition of a progressing site in our study: sites which are categorised as progressing are not all in a state of disease progression at the moment of GCF sampling. As well, sites that do not fill the criteria of a progressing site are categorised as stable though active disease phases in these sites may be going on. Disease progression is episodic, occurs only infrequently and is slow in most chronic periodontitis patients. During a study period it is likely that only a small number of sites with attachment loss can be confirmed (Chambers *et al.* 1991). In addition, only a small group of periodontitis patients manifest multiple progressing sites (Persson *et al.* 1995). The nature of chronic periodontitis is that "sick" sites are not sick all the time. Part of the sites may be in quiescence at the particular moment. This makes the calculation of sensitivity difficult and gives a reason to conclude that repeatedly positive test results seemingly indicate sites at risk of disease progression. Sensitivity was rather poor when it was calculated for sites regarded as refractory (high-responder) sites and stable (low-responder) sites (*V*) though refractory sites are possibly in a more constant state of instability and chronically "sick" which are well reflected in MMP-8 GCF concentrations (*V*).

Repeatedly positive test results in non-smokers' sites during the maintenance phase may also indicate a site at risk. However, the cut-off value of our test, 1000 µg/l of MMP-8, may be too low to be valuable in non-smokers. In non-smokers' sites MMP-8 concentrations remained in most cases after SRP, above the concentration of the cut-off value of the test. For non-smokers' a new cut-off value should be defined. With the current cut-off value the test

seems to be useful in smokers' sites and it recognises both sites at risk of attachment loss progression and sites/patients suffering of refractory periodontitis.

Moreover, with regards to the different levels of MMP-8 concentrations in GCF from healthy or gingivitis subjects and those with periodontitis (*IV*), the current cut-off value of the test stick may be useful in differentiating incipient periodontitis or gingivitis at risk to proceed to periodontitis. Hanioka *et al.* (2005) studied in a cross-sectional setting different combinations of periodontal biomarkers which could predict periodontitis, i.e. which could find persons with minor periodontitis changes. None of the studied biomarkers alone (haemoglobin, albumin, transferring, α1-AT, fibronectin, IgA, IgG, IgM, lactoferrin, myeloperoxidase, neutrophil elastase) in GCF was able to identify subjects with periodontal changes but GCF neutrophil elastase and IgA in combination seemed to afford prediction of periodontal disease. Thus, it cannot be excluded that the best predictor of progression of periodontitis will be a combination of biomarkers.

Our MMP-8 chair-side dipstick test and the antibody used in IFMA are not completely selective or specific for the active form of MMP-8 (*II*); it detects also to some extent the latent forms of MMP-8 (Hanemaaijer *et al.* 1997). A MMP-8 dipstick test for monitoring periodontal stability could be more useful if it were supplied with a MMP-8 antibody being selective and specific for the active form of MMP-8. Especially the elevation of the active form of MMP-8 in GCF has been shown to be associated with both conversion of gingivitis to periodontitis and the progression of periodontitis (Lee *et al.* 1995a, b; Mancini *et al.* 1999; Romanelli *et al.*1999; Pozo *et al.* 2005). Nonetheless, since MMP-8 (as well as other MMPs) are not anymore solely regarded as tissue destructive proteinases but also anti-inflammatory modifiers of host and immunoresponses (Sorsa *et al.* 2004a, b) their levels and assessment from GCF (*II, IV, V*) may not be interpreted to indicate tissue destruction alone but also protective inflammation/immune responses (Owen *et al.* 2004; Sorsa *et al.* 2004a; Gueders *et al.* 2005). Future studies are required to address these aspects. Our dip-stick can be modified to detect also other biomarkers with known monoclonal antibodies. Thus the value of simultaneous detection of several biomarkers by means of more than one specific dip-stick should be considered.

9.3. *CHLAMYDIA PNEUMONIAE* IN PERIODONTAL LESIONS (*VI*)

The detection method for *C. pneumoniae* which was used in our study is very sensitive and has been among the best in international comparison of PCR methods (Rintamäki *et al.* 2002). The sensitivities of PCR methods for *C. pneumoniae* seem to vary hugely (Boman *et al.* 1999; Apfalter *et al.* 2002).

C. pneumoniae cannot be regarded as periodontopathogen and, this far, it has not been identified from the oral cavity. The *C. pneumoniae* positive patient of our study did not differ with regard to periodontal parameters or GCF MMP-8 concentrations from other study subjects, and the positive count turned negative after SRP treatment. Nevertheless, our finding suggests that severe periodontal lesions can harbour *C. pneumoniae* (*VI*). Deep periodontal pockets may serve as a reservoir for pathogens which are not regarded as participating in the pathogenesis of periodontitis or other oral diseases but which have relevance with the systemic health status. The finding may have relevance because of the association of dental infections and atherosclerosis. Oral inflammation may promote the formation of atherosclerosis and thrombosis by elevation of blood levels of fibrinogen, leukocytes, clotting factors, and cytokines, and by alteration of the metabolism and functions of endothelial cells and monocytes/macrophages. Low-grade infections may be one cause behind the inflammatory reaction observed in atherosclerotic lesions and acute ischemic symptoms (Mattila *et al.* 1998). The potential role of the oral cavity as a reservoir of *C. pneumoniae* should be repeated with a large number of periodontitis patients and to be compared with periodontally healthy subjects.

10 CONCLUSIONS

1. The immunoassay for MMP-8 is a specific and convenient method for chair-side GCF enzyme analysis in respect to the functional enzyme assays which at present are not specific for individual MMPs.
2. MMP-8 levels in GCF in untreated periodontitis patients decrease with scaling and root planning (SRP) and are associated with improvement in clinical periodontal parameters. During successful maintenance MMP-8 concentrations can even further reduce until the wound healing phase of periodontal tissues is over.
3. GCF concentration of MMP-8 and MMP-8 specific chair-side dipstick test may be useful for monitoring the treatment (SRP) response of periodontitis.
4. GCF MMP-8 concentrations and MMP-8 testing differentiates periodontally healthy subjects, subjects with gingivitis and subjects with periodontitis.
5. Despite on average lower MMP-8 levels in smokers' GCF, MMP-8 concentrations in smokers' deteriorating sites are at a similar level as in non-smokers' corresponding sites. The highest GCF MMP-8 levels can be found from GCF of smokers with poor treatment response to conventional periodontal treatment (SRP) and instability during the maintenance phase.
6. In non-smokers, pre-treatment (baseline) GCF MMP-8 concentrations decrease after treatment but remain higher than in healthy individuals' or in gingivitis patients' GCF.
7. A repeatedly positive MMP-8 test result recognises periodontitis patients with a poor treatment response, i.e. refractory periodontitis, and sites at risk of progression of periodontal attachment loss especially in smokers. For non-smokers the cut-off value of the chair-side test stick (1000 μg/l) should be further adjusted.
8. Gingivitis patients with repeatedly positive MMP-8 test results may be those who are at risk of periodontitis or are possibly already undergoing irreversible minor changes.
9. In the future a corresponding MMP-8 dip-stick test for monitoring periodontal stability should be supplied with a MMP-8 antibody selective and specific for the active forms of MMP-8.
10. *Chlamydia pneumoniae* can harbour subgingival plaque of a periodontal lesion though it is not normally involved in periodontitis.

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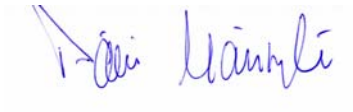

Päivi Mäntylä

The witticism which my husband Juha chose and insisted on adding is by Jouni Lompolo, alias Origo:

Hampaattomuuden aiheuttamaa haittaa aiheettomasti liioitellaan; tärkeintähän on sentään kaunis sielu.

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